

CORTICOTROPIN-RELEASING FACTOR₂ RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of United States Application No. 09/881,401, filed June 13, 2002, now issued as U.S. Patent No. 6,723,841; which is continuation of United States Application No. 09/227,482, filed January 7, 1999, now abandoned; which is a continuation of pending United States Patent Application No. 08/485,984, filed June 7, 1995, now abandoned; which is a continuation-in-part of pending United States Patent Application No. 08/381,433, filed January 31, 1995, now issued as U.S. Patent No. 5,786,203; which is a continuation-in-part of United States Patent Application No. 08/259,959, filed June 14, 1994, now abandoned.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cell surface receptors, and more specifically, to Corticotropin-Releasing Factor₂ receptors.

Description of the Related Art

Corticotropin-releasing factor ("CRF") is a 41-amino acid peptide originally isolated from the hypothalamus by virtue of its ability to stimulate the production of adrenocorticotrophic hormone ("ACTH") and other proopiomelanocortin ("POMC") products of the anterior pituitary (Vale et al., *Science* 213:1394-1397, 1981). Briefly, CRF is believed to initiate its biological effects by binding to a plasma membrane receptor which is distributed throughout the brain (DeSouza et al., *Science* 224:1449-1451, 1984), pituitary (Wynn et al., *Biochem. Biophys. Res. Comm.* 110:602-608, 1983), adrenals (Udelsman et al., *Nature* 319:147-150, 1986) and spleen (Webster, E.L., and E.B. DeSouza, *Endocrinology* 122:609-617, 1988). This receptor is coupled to a GTP-binding protein (Perrin et al., *Endocrinology* 118:1171-1179, 1986) which

mediates CRF-stimulated increase in intracellular cAMP (Bilezikjian, L.M., and W.W. Vale, *Endocrinology* 113:657-662, 1983).

In addition to its role in stimulating the production of ACTH and POMC, CRF is also believed to coordinate many of the endocrine, autonomic, and behavioral responses to stress, and may be involved in the pathophysiology of affective disorders. Moreover, CRF is believed to be a key intermediary in communication between the immune, central nervous, endocrine and cardiovascular systems (Crofford et al., *J. Clin. Invest.* 90:2555-2564, 1992; Sapolsky et al., *Science* 238:522-524, 1987; Tilders et al., *Regul. Peptides* 5:77-84, 1982; Fisher et al., *Reg. Peptide* 5:153-161, 1983).

A receptor for CRF has been cloned from rat (Perrin et al., *Endo* 133(6):3058-3061, 1993), and human brain (Chen et al., *PNAS* 90(19):8967-8971, 1993; Vita et al., *FEBS* 335(1):1-5, 1993). This receptor is a 415 amino acid protein comprising seven membrane spanning domains, and has a predicted molecular weight of 44,000 daltons. A comparison of identity between rat and human sequences shows a high degree of homology (97%) at the amino acid level. In addition, Scatchard analysis of recombinantly produced human receptor demonstrates a single component site, with a high affinity (K_d of 1.6 ± 0.3 nM) for CRF.

The present invention provides new, previously unidentified CRF receptors, designated as the "Corticotropin-Releasing Factor-2 ("CRF₂"), Corticotropin-Releasing Factor receptors. In addition, the present invention provides compositions and methods which utilize such CRF₂ receptors, as well as other, related advantages.

BRIEF SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods which utilize CRF₂ receptors (also termed "CRF₂ Corticotropin-Releasing Factor receptors" or "CRF₂R"). Within one aspect of the present invention, isolated nucleic acid molecules are provided which encode CRF₂ receptors. Within one embodiment, nucleic acid molecules are provided which encode a CRF₂ receptor such as that disclosed in Sequence I.D. No. 4, from amino acid number 1 to amino acid number 411.

Within another embodiment, nucleic acid molecules are provided which comprise the sequence of nucleotides in Sequence I.D. No. 3, from nucleotide number 216 to nucleotide number 1449. Within another embodiment, nucleic acid molecules are provided which encode a CRF₂ receptor such as that disclosed in Sequence ID No. 2 from amino acid number 1 to amino acid number 431. Within another embodiment, nucleic acid molecules are provided which comprise the sequence of nucleotides in Sequence ID No. 1 from nucleotide number 44 to nucleotide number 1336. Nucleic acid molecules which encode CRF₂ receptors of the present invention may be isolated from virtually any warm-blooded animal, including for example, humans, macaques, horses, cattle, sheep, pigs, dogs, cats, rats and mice.

Within another aspect of the present invention, isolated nucleic acid molecules are provided which encode portions of a CRF₂ receptor, such as the N-terminal extracellular domain. Within one embodiment, isolated nucleic acid molecules are provided comprising the sequence of nucleotides in Sequence I.D. No. 3, from nucleotide number 216 to nucleotide number 570. Within another embodiment, isolated nucleic acid molecules are provided which encode a protein having the amino acid sequence of Sequence I.D. No. 4, from amino acid number 1 to amino acid number 118. Within another embodiment, isolated nucleic acid molecules are provided comprising the sequence of nucleotides in Sequence ID No. 1 from nucleotide number 44 to nucleotide number 451. Within another embodiment, isolated nucleic acid molecules are provided which encode a protein having the amino acid sequence of Sequence ID No. 2 from amino acid number 1 to amino acid number 138.

Within other aspects of the invention, expression vectors are provided which are capable of expressing the above-described nucleic acid molecules. Within other aspects, recombinant viral vectors are provided which are capable of directing the expression of the above-described nucleic acid molecules. Representative examples of such viral vectors include retroviral vectors, adenoviral vectors, and herpes simplex virus vectors. Also provided by the present invention are host cells which contain the above-described expression vectors, as well as the receptor or portions thereof which

are encoded by the above-described nucleic acid molecules. Within other embodiments, isolated portions of CRF₂ receptors are provided, including for example, isolated portions of extracellular domains such as the N-terminal extracellular domain.

Within other aspects of the invention, isolated antibodies are provided which are capable of specifically binding to the above-described CRF₂ receptors. Within one embodiment, the antibodies may be selected from the group consisting of polyclonal antibodies, monoclonal antibodies, and antibody fragments. Within other embodiments, antibodies are provided which are capable of blocking the binding of CRF (or other substrates such as sauvagine or urotensin I) to a CRF₂ receptor. Within preferred embodiments, the antibodies may be selected from the group consisting of murine and human antibodies. Within preferred aspects of the invention, the above-noted antibodies are produced by hybridomas.

Within yet another aspect of the present invention, nucleic acid molecules are provided which are capable of specifically hybridizing to a nucleic acid molecule encoding any of the CRF₂ receptors described above. Such molecules may be between at least "y" nucleotides long, wherein "y" is any integer between 14 and 1230, and furthermore, may be selected suitable for use as probes or primers described below. Particularly preferred probes of the present invention are at least 18 nucleotides in length.

Within other aspects of the present invention, methods for detecting the presence of a compound which binds to a CRF₂ receptor are provided, comprising the steps of (a) exposing one or more compounds to cells that express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compounds to the receptors, and (b) isolating compounds which bind to the receptors, such that the presence of a compound which binds to a CRF₂ receptor may be detected. Within another aspect, methods for detecting the presence of a compound which binds to a CRF₂ receptor are provided, comprising the steps of (a) exposing one or more compounds to a CRF₂ receptor N-terminal extracellular domain under conditions and for a time sufficient to allow binding of a compound to the N-terminal extracellular

domain, and (b) isolating compounds which bind to the CRF₂ receptor N-terminal extracellular domain, such that the presence of a compound which binds to a CRF₂ receptor may be detected. Within one embodiment, the compounds are labeled with an agent selected from the group consisting of fluorescent molecules, enzymes, and radionuclides.

Within other aspects of the present invention, methods for determining whether a selected compound is a CRF₂ receptor agonist or antagonist are provided, comprising the steps of (a) exposing a selected compound to cells which express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compound and an associated response in intracellular levels of cAMP, and (b) detecting either an increase or decrease in the level of intracellular cAMP, and thereby determining whether the selected compound is a CRF₂ receptor agonist or antagonist.

Within other aspects, methods are provided for detecting the presence of a CRF₂ receptor agonist or antagonist in a pool of compounds, comprising the steps of (a) exposing a pool of compounds to cells which express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compound and an associated response in intracellular levels of cAMP, and (b) isolating compounds which either increase or decrease the intracellular level of cAMP, such that the presence of a CRF₂ receptor agonist or antagonist may be detected.

Within another aspect, methods for determining whether a selected compound is a CRF₂ receptor antagonist are provided, comprising the steps of (a) exposing a selected compound in the presence of a CRF₂ receptor agonist to a recombinant CRF₂ receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the compound to the receptor and an associated response through the pathway, and (b) detecting a reduction in the stimulation of the response pathway resulting from the binding of the compound to the CRF₂ receptor, relative to the stimulation of the response pathway by the CRF₂ receptor agonist alone, and therefrom determining the presence of a CRF₂ antagonist. Within other aspects, methods are provided for determining whether a selected compound is a CRF₂ receptor

agonist, comprising the steps of (a) exposing a selected compound to a recombinant CRF₂ receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the compound to the receptor and an associated response through the pathway, and (b) detecting an increase in stimulation of the response pathway resulting from the binding of the compound to the CRF₂ receptor, and therefrom determining the presence of a CRF₂ receptor agonist.

Within other aspects of the present invention, methods are provided for treating CRF₂ receptor-associated diseases, wherein it is desired to either increase or decrease stimulation of a CRF₂ receptor response pathway. For example, within one aspect methods are provided for treating cerebrovascular disorders such as stroke, reperfusion injury and migraines, comprising the step of administering to a patient a therapeutically effective amount of a CRF₂ receptor antagonist, such that the disorder is remedied or alleviated. Within other aspects, methods are provided for treating learning or memory disorders, comprising administering to a patient a therapeutically effective amount of a CRF₂ receptor antagonist. Within yet other aspects, methods are provided for treating Alzheimer disease, comprising administering to a patient a therapeutically effective amount of a CRF₂ receptor antagonist. Representative examples of suitable CRF₂ receptor antagonist include α -helical oCRF (9-41), or d-Phe r/h CRF (12-41).

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates the structure of one representative CRF₂ receptor (CRF_{2 β} ; Sequence ID No. 2).

Figure 2 schematically illustrates the structure of a second representative CRF₂ receptor (CRF_{2α}; Sequence ID No. 4).

Figure 3 is a graph which depicts cAMP accumulation in cells transfected with the CRF₁ receptor.

Figure 4 is a graph which depicts cAMP accumulation in cells transfected with the CRF₂ receptor.

Figure 5 is a graph which depicts the effect of the antagonists α -helical and d-Phe on Sauvagine stimulated cAMP production in CRF₁ transfected cells.

Figure 6 is a graph which depicts the effect of the antagonists α -helical and d-Phe on Sauvagine stimulated cAMP production in CRF_{2α} transfected cells.

Figure 7 is a photograph of an RNase protection assay of two CRF₂ subtypes (CRF_{2α}, and CRF_{2β}), and β -actin. Ts=testis; Ht=heart; Sk=skeletal muscle; Lv=liver; Kd=kidney; Sp=spleen; Ol=olfactory bulb; St=striatum; Hy=hypothalamus; Pt=pituitary; Cx=cortex; Hp=hippocampus.

Figures 8A, A', B, B', C and C' are a series of photographs which show the anatomical distribution of two CRF₂ subtypes. 8A, B and C are coronal sections of rat brain probed with CRF_{2α} and CRF_{2β}. Figure 8A', B' and C' are adjacent sections probed with CRF_{2β} antisense cRNA.

Figure 9 is a schematic illustration of nucleotide sequence homology between CRF₁ and CRF₂ receptors across the coding region of the receptors. Lower bars indicate the region of the receptors against which CRF₁ and CRF₂ cRNA probes were designed.

Figure 10 is two color-coded digitized images of CRF₁ and CRF₂ receptor mRNA expression in adjacent horizontal brain sections. Regions exhibiting high levels of mRNA expression are coded in red and orange while the lowest levels of expression are coded in blue.

Figures 11A, B, C, D and E are a series of photographs which show the rostro-caudal (A-E) distribution of CRF₂ receptor mRNA (left hemisphere) and CRF₁ receptor mRNA (right hemisphere) in digitized coronal brain sections. Epy, ependymal

layer of olfactory bulb; Int Gr, internal granule cell layer of olfactory bulb; G1, granule cell layer of olfactory bulb; LSI, lateral septal nucleus (intermediate part); LSV, lateral septal nucleus (ventral part); MS, medial septal nucleus; Fr Ctx, frontal cortex; Pir, piriform cortex; CA₁, field CA₁ (Ammons horn); CA₃, field CA₃ (Ammons horn); DG, dentate gyrus; Chp, choroid plexus; MeA, medial amygdaloid nucleus; VMH, ventromedial hypothalamic nucleus; Cing Ctx, cingulate cortex; BLA, basolateral amygdaloid nucleus; PCo, posterior cortical amygdaloid nucleus; RN, red nucleus; Oc Ctx, occipital cortex; MG, medial geniculate nucleus; PDTg, posterior dorsal tegmental nucleus; Trg Nuc, trigeminal nuclei; Pn, pontine gray.

Figures 12A and B are two darkfield photomicrographs of cells hybridized with (³⁵S) cRNA CRF₂ probe in (A) the intermediate (LSI) and ventral (LSV) lateral septal nuclei. At high resolution (B) note the high level of CRF₂ receptor expression in cells in the ventral lateral septum. Cau, caudate; LV, lateral ventricle.

Figures 13A, B and C are a series of darkfield photomicrographs of cells hybridized with (A) (³⁵S) cRNA CRF probe, (B) (³⁵S) cRNA CRF₂ probe and (C) (³⁵S) cRNA CRF₁ probe in adjacent coronal sections through the bed nucleus of the stria terminalis (BNST). In (A) note the higher concentration of CRF-expressing cells in the posterolateral area (BNSTpl) while in (B) CRF₂ receptor expression is predominantly localized to the medial aspects of the nucleus (BNSTpm). In (C) CRF₁ receptor mRNA expression is evident in both medial and lateral aspects of the nucleus. Fx, fornix.

Figures 14A and B are two darkfield photomicrographs of cells hybridized with (A) (³⁵S) cRNA CRF₂ probe and (B) (³⁵S) cRNA CRF₁ probe in the anterior cortical amygdaloid nucleus (ACo). In (A) note the high level of CRF₂ receptor mRNA expression in cells throughout the nucleus while in (B) CRF₁ receptor expression is comparable to background signal. CRF₂ receptor mRNA expression is also evident within the supraoptic nucleus in (A), an area where CRF₁ receptor expression is undetectable (B). opt, optic tract, arrows in (A) indicate section edge.

Figures 15A, B and C are a series of darkfield photomicrographs of cells hybridized with (A) (³⁵S) cRNA CRF₁ probe and (B) and (C) (³⁵S) cRNA CRF₂ probe in

the hippocampal formation. Within dorsal hippocampus, both CRF₁ and CRF₂ receptor expression was relatively low. However, within the ventral hippocampus (C), cells expressing high levels of CRF₂ receptor mRNA were evident in the dentate gyrus and subiculum. *, emulsion artifact.

Figures 16A and B are two darkfield photomicrographs of cells hybridized with (³⁵S) cRNA CRF₂ probe in the ventromedial hypothalamic nucleus (VMH) (A) and (B). At high resolution (B), note the high level of CRF₂ receptor expression in both the dorsomedial (DM) and ventrolateral (VL) aspects of the nucleus. 3v, third ventricle; opt, optic tract.

Figures 17A, B, C and D are a series of darkfield photomicrographs of cells hybridized with (³⁵S) cRNA CRF₂ probe (A) and (C) and (³⁵S) cRNA CRF₁ probe (B) and (D) in adjacent coronal sections through the supraoptic nucleus (SO) and suprachiasmatic nucleus (Sch). Note the absence of CRF₁ receptor expression in either nucleus (B) and (D). Opt, optic tract; *, labeled arteriole.

Figures 18A, B and C are a series of darkfield photomicrographs of cells hybridized with (A) (³⁵S) cRNA CRF probe, (B) (³⁵S) cRNA CRF₂ probe and (C) (³⁵S) cRNA CRF₁ probe in adjacent sections through the paraventricular nucleus of the hypothalamus. In (A), CRF expressing cells are evident throughout the medial and dorsal aspects of the paraventricular nucleus. In (B) note that CRF₂ receptor expression is most prominent in the medial parvocellular area (mpv) with only scattered labeled cells evident in the dorsal sub-division. CRF₁ receptor expression is unremarkable in both sub-divisions (C). 3v, third ventricle; Fx, fornix.

Figures 19A, B and C are a series of darkfield photomicrographs of cells hybridized with (³⁵S) cRNA CRF₂ probe in (A) dorsal raphe (DR) and central gray (CG), (B) median raphe (MnR) and (C) interpeduncular nucleus (IPN).

Figure 20A is a photomicrograph which shows CRF₂ receptor mRNA expression in the choroid plexus (ChP). Figure 20B is a photomicrograph which shows an adjacent nissl stained section. 4v, fourth ventricle.

Figure 21A is a high power darkfield image of CRF₂ receptor mRNA expression in a cerebral arteriole. Figure 21B is a brightfield nissl-stained section of arteriole shown in (A). Note the characteristic muscular arteriole wall in (B).

Figures 22A, B, C and D are a series of darkfield photomicrographs of CRF₁ receptor mRNA expression (A) and (B) and CRF₂ receptor mRNA expression (C) and (D) in the anterior lobe (AL) of the pituitary gland. In (A), note the clustering of cells expressing CRF₁ mRNA presumably reflecting the distribution of pituitary corticotropes while CRF₂ mRNA expression is present only in scattered cells (C). At high resolution, prominent accumulation of silver grains are evident over anterior lobe cells hybridized with (³⁵S) cRNA CRF₁ probe, arrow in (B), while only weak accumulations of silver grains were evident over cells hybridized with (³⁵S) cRNA CRF₂ probe, arrow in (D).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

"CRF₂ receptors" (also termed "CRF₂ Corticotropin-Releasing Factor receptors" or "CRF₂R") as utilized herein refers to receptor proteins which bind corticotropin-releasing factor and other proteins such as urotensin I and sauvagine. CRF₂ receptors may be distinguished from other receptors such as Corticotropin-Releasing Factor receptors based upon criteria such as affinity of substrate binding, tissue distribution, and sequence homology. For example, CRF₂ receptors of the present invention should be greater than 70% homologous, preferably greater than 75% to 80% homologous, more preferably greater than 85% to 90% homologous, and most preferably greater than 92%, 95%, or 97% homologous to the CRF₂ receptors disclosed herein (e.g., Sequence I.D. No. 3). In their native configuration, CRF₂ receptors are believed to exist as membrane bound proteins, consisting of an N-terminal extracellular domain, seven transmembrane domains separated by three

intracellular and three extracellular loops, and a C-terminal intracellular domain (see Figures 1 and 2). As utilized within the context of the present invention CRF₂ receptors should be understood to include not only the proteins which are disclosed herein (see Sequence I.D. Nos. 2, 4 and 8), but substantially similar derivatives and analogs as discussed below.

"Nucleic acid molecule" refers to a nucleic acid sequence, in the form of a separate fragment or as a component of a larger nucleic acid construct, which has been derived from nucleic acid isolated at least once in substantially pure form, *i.e.*, free of contaminating endogenous materials, and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic nucleic acid containing the relevant sequences may also be used. Sequences of non-translated nucleic acid may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Recombinant expression vector" refers to a replicable nucleic acid construct used either to amplify or to express nucleic acid sequences which encode CRF₂ receptors. This construct comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences.

As noted above, the present invention provides isolated nucleic acid molecules encoding CRF₂ receptors. Briefly, CRF₂ receptors are G-coupled protein receptors, which are capable of binding a substrate (such as CRF, or other substrates such as sauvagine and urotensin I), and transducing the signal provided by the substrate to the cell. Such signal transduction typically occurs when a response

pathway is activated by an external stimulus that is generally, but not always, directly coupled to a membrane-bound receptor. Response pathways generally induce cellular responses such as extracellular matrix secretion from responsive cell lines, hormone secretion, chemotaxis, differentiation, or the initiation or inhibition of cell division of responsive cells. As used herein, the coupling of receptors to response pathways refers to the direct activation of a response pathway or the transduction of a signal via a second messenger, such as a G-protein, to activate a cellular response pathway.

One representative CRF₂ receptor which may be obtained utilizing the methods described herein (see Example 1) is schematically illustrated in Figure 1 (see also Figure 2. Briefly, this CRF₂ receptor is composed of an Extracellular N-terminal Domain (amino acids 1 - 117), a first Transmembrane Domain (amino acids 118 - 138), a first Intracellular Domain (139 - 147), a second Transmembrane Domain (148 - 167), a second Extracellular Domain (168 - 184), a third Transmembrane Domain (185 - 208), a second Intracellular Domain (229 - 223), a fourth Transmembrane Domain (224 - 244), a third Extracellular Domain (245 - 261), a fifth Transmembrane Domain (262 - 286), a third Intracellular Domain (287 - 309), a sixth Transmembrane Domain (310 - 329), a fourth Extracellular Domain (330 - 342), a seventh Transmembrane Domain (343 - 363), and a C-terminal Intracellular Domain (364 - 411).

Although the above CRF₂ receptor has been provided for purposes of illustration (see also Figure 2 and Sequence I.D. No. 2), the present invention should not be so limited. In particular, the present invention provides a wide variety of additional CRF₂ receptors which have substantial similarity to the sequences disclosed in Sequences I.D. Nos. 1-4. As utilized within the context of the present invention, nucleic acid sequences which encode CRF₂ receptors are deemed to be substantially similar to those disclosed herein if: (a) the nucleic acid sequence is derived from the coding region of a native CRF₂ receptor gene (including, for example, allelic variations of the sequences disclosed herein); (b) the nucleic acid sequence is capable of hybridization to nucleic acid sequences of the present invention under conditions of either moderate (e.g. 50% formamide, 5 x SSPE, 5 x Denhardt's, 0.1% SDS, 100 ug/ml

Salmon Sperm nucleic acid, and a temperature of 42°C) or high stringency (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, NY, 1989); or (c) nucleic acid sequences are degenerate as a result of the genetic code to the nucleic acid sequences defined in (a) or (b). Furthermore, although nucleic acid molecules are primarily referred to herein, as should be evident to one of skill in the art given the disclosure provided herein, a wide variety of related nucleic acid molecules may also be utilized in various embodiments described herein, including for example, RNA, nucleic acid analogues, as well as chimeric nucleic acid molecules which may be composed of more than one type of nucleic acid.

In addition, as noted above, within the context of the present invention "CRF₂ receptors" should be understood to include derivatives and analogs of the CRF₂ receptors described above. Such derivatives include allelic variants and genetically engineered variants that contain conservative amino acid substitutions and/or minor additions, substitutions or deletions of amino acids, the net effect of which does not substantially change the biological activity (e.g., signal transduction) or function of the CRF₂ receptor. Such derivatives are generally greater than about 70% to 75% similar to the corresponding native CRF₂ receptor, preferably greater than 80% to 85% similar, more preferably greater than 90% to 95% similar, and most preferably greater than 97% similar. Percent similarity may be determined, for example, by comparing sequence information with the GAP program, which utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482, 1981). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (*Nucl. Acids Res.* 14:6745, 1986), as described by Schwartz and Dayhoff (ed., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979); (2) a penalty

of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

The primary amino acid structure of CRF₂R receptors may also be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Covalent derivatives may be prepared by linking particular functional groups of CRF₂R amino acid side chains or at the N- or C-termini. Other derivatives of CRF₂R within the scope of this invention include covalent or aggregative conjugates of CRF₂R or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α -factor leader). CRF₂R protein fusions may also comprise peptides added to facilitate purification or identification of CRF₂R (e.g., poly-His, which allows purification of the protein via, for example, a NTA nickel-chelating column), or FLAG (Hopp et al., *Bio/Technology* 6:1204-1210, 1988). Other useful fusion proteins include luciferase, beta-galactosidase (Grey et al., *PNAS* 79:6598, 1982), trp E. (Itakura et al., *Science* 98:1056, 1977), and protein A (Uhlen et al., *Gene* 23:369, 1983). Within preferred embodiments, fusion proteins may include a site that is specifically recognized and cleaved, for example, by a collagenase (which cleaves x in the sequence Pro-x-Gly-Pro, where x is a neutral amino acid; see Keil et al., *FEBS Letters* 56:292-296, 1975). Factor Xa (which cleaves after arginine in the sequence Ile-Glu-Gly-Arg; see Nogai et al., *Methods Enzymol.* 153:461-481, 1987).

The present invention also includes CRF₂R proteins with or without associated native-pattern glycosylation. Briefly, CRF₂R expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or significantly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of CRF₂R nucleic acids in bacteria

such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of mammalian CRF₂R having inactivated N-glycosylation sites may be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins may be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are generally characterized by the amino acid triplet Asn-A₁-Z, where A₁ is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A₁ and Z, or an amino acid other than Asn between Asn and A₁.

Proteins which are biologically active and substantially similar to CRF₂R proteins may also be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted or replaced with other amino acids to prevent formation of incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively, *i.e.*, the most preferred substitute amino acids are those having physicochemical characteristics resembling those of the residue to be replaced.

When a substitution, deletion, or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered utilizing, for example, a binding assay such as that disclosed within the Examples. Particularly preferred regions wherein mutations, deletions, substitutions, or insertions may be accomplished include those which are not directly involved with binding of the receptor to its ligand. Such regions include all portions of the receptor, except the N-terminal extracellular domain, as well as the third intracellular loop between Th5 and Th6 (see *e.g.*, Kobilka, *Ann. Rev. Neuro.* 15:87-114, 1992).

Mutations in nucleotide sequences constructed for expression of proteins which are substantially similar to CRF₂ receptors should preferably preserve the reading frame phase of the coding sequences. Furthermore, the mutations should preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at a target codon, or across a given site and the expressed CRF₂R mutants screened for the biological activity.

Not all mutations in the nucleotide sequence which encodes CRF₂R will be expressed in the final product. For example, nucleotide substitutions may be made to enhance expression primarily to avoid secondary structure loops in the transcribed mRNA, or to provide codons that are more readily translated by the selected host, e.g., the well-known, *E. coli* preference codons for *E. coli* expression.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Representative methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik, *Bio Techniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Sambrook et al. (*Molecular cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, 1989); and U.S. Patent Nos. 4,518,584 and 4,737,462.

CRF₂ receptors, as well as substantially similar derivatives or analogs may be used as therapeutic reagents, immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of CRF, sauvagine, urotensin I, other related molecules, or other binding ligands such as anti-CRF₂ receptor antibodies. Moreover, CRF₂ receptors of the present invention may be utilized to screen compounds for CRF₂ receptors agonist or antagonistic activity. CRF₂ receptor proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromine-activated, bisoxirane-activated, carbonyldiimidazole-activated, or tosyl-activated, agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, CRF₂R may be used to selectively bind (for purposes of assay or purification) anti-CRF₂R antibodies or CRF.

Isolation of CRF₂ Receptor cDNA Clones

As noted above, the present invention provides isolated nucleic acid molecules which encode CRF₂ receptors. Briefly, nucleic acid molecules which encode CRF₂ receptors of the present invention may be readily isolated from a variety of warm-blooded animals, including for example, humans, macaques, horses, cattle, sheep, pigs, dogs, cats, rats and mice. Particularly preferred tissues from which nucleic acid molecules which encode CRF₂ receptors may be isolated include brain and neural tissues such as the hypothalamus, hippocampus, and frontal cortex, as well as other tissues such as the lung, heart, skeletal muscle, or kidney. Nucleic acid molecules which encode CRF₂ receptors of the present invention may be readily isolated from conventionally prepared cDNA libraries (see, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, NY, 1989) or from commercially obtained libraries (e.g., Stratagene, LaJolla, Calif.) utilizing the disclosure provided herein. Particularly preferred methods for obtaining isolated nucleic acid molecules which encode CRF₂ receptors of the present invention are described in more detail below in Example 1 (see also Sequence I.D. Nos. 1 and 3).

As noted above, within particularly preferred embodiments of the invention, isolated nucleic acid molecules are provided which encode human CRF₂ receptors. Briefly, such nucleic acid molecules may be readily obtained by probing a human cDNA library either with a specific sequence as described below in Example 1, or with a rat sequence (e.g., Sequence I.D. Nos. 1 or 3) under conditions of low stringency (e.g., 35% formamide, 5 x SSC, 5x Denharts, 0.1% SDS, 100 ug/ml salmon sperm nucleic acid, at 42°C for 12 hours. This may be followed by extensive washing with 2x SSC containing 0.2% SDS at 50°C. Suitable cDNA libraries may be obtained from commercial sources (e.g., Stratagene, LaJolla, Calif.), or prepared utilizing standard techniques (see, e.g. Sambrook et al., *supra*).

Production of Recombinant CRF₂ Receptors

As noted above, the present invention also provides recombinant expression vectors which include synthetic or cDNA-derived nucleic acid fragments encoding CRF₂ receptors or substantially similar proteins, which are operably linked to suitable transcriptional or translation regulatory elements derived from mammalian, microbial, viral or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and, within preferred embodiments, sequences which control the termination of transcription and translation. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. Nucleic acid regions are operably linked when they are functionally related to each other. For example, nucleic acid for a signal peptide (secretory leader) is operably linked to nucleic acid for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked

means contiguous and, in the case of secretory leaders, contiguous and in reading frame.

Expression vectors may also contain nucleic acid sequences necessary to direct the secretion of a polypeptide of interest. Such nucleic acid sequences may include at least one secretory signal sequence. Representative examples of secretory signals include the alpha factor signal sequence (pre-pro sequence; Kurjan and Herskowitz, *Cell* 30:933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake, EP 116,201), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670), the SUC2 signal sequence (Carlson et al., *Mol. Cell. Biol.* 3:439-447, 1983), the α -1-antitrypsin signal sequence (Kurachi et al., *Proc. Natl. Acad. Sci. USA* 78:6826-6830, 1981), the β -2 plasmin inhibitor signal sequence (Tone et al., *J. Biochem. (Tokyo)* 102:1033-1042, 1987), the tissue plasminogen activator signal sequence (Pennica et al., *Nature* 301:214-221, 1983), the *E. coli* PhoA signal sequence (Yuan et al., *J. Biol. Chem.* 265:13528-13552, 1990) or any of the bacterial signal sequences reviewed, for example, by Oliver (*Ann. Rev. Microbiol.* 39:615-649, 1985). Alternatively, a secretory signal sequence may be synthesized according to the rules established, for example, by von Heinje (*Eur. J. Biochem.* 133:17-21, 1983; *J. Mol. Biol.* 184:99-105, 1985; *Nuc. Acids Res.* 14:4683-4690, 1986).

For expression, a nucleic acid molecule encoding a CRF₂ receptor is inserted into a suitable expression vector, which in turn is used to transform or transfect appropriate host cells for expression. Host cells for use in practicing the present invention include mammalian, avian, plant, insect, bacterial and fungal cells. Preferred eukaryotic cells include cultured mammalian cell lines (e.g., rodent or human cell lines) and fungal cells, including species of yeast (e.g., *Saccharomyces* spp., particularly *S. cerevisiae*, *Schizosaccharomyces* spp., or *Kluyveromyces* spp.) or filamentous fungi (e.g., *Aspergillus* spp., *Neurospora* spp.). Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred. Methods for producing recombinant proteins in a variety of prokaryotic and eukaryotic host cells are generally known in the art (see

"Gene Expression Technology," *Methods in Enzymology*, Vol. 185, Goeddel (ed.), Academic Press, San Diego, Calif., 1990; see also, "Guide to Yeast Genetics and Molecular Biology," *Methods in Enzymology*, Guthrie and Fink (eds.) Academic Press, San Diego, Calif., 1991). In general, a host cell will be selected on the basis of its ability to produce the protein of interest at a high level or its ability to carry out at least some of the processing steps necessary for the biological activity of the protein. In this way, the number of cloned nucleic acid sequences which must be transfected into the host cell may be minimized and overall yield of biologically active protein may be maximized.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., *Proc. Natl. Acad. Sci. USA* 76:1035-1039, 1978), YEpl3 (Broach et al., *Gene* 8:121-133, 1979), POT vectors (Kawasaki et al., U.S. Patent No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, *Nature* 275:104-108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include *LEU2* (Broach et al., *ibid.*), *URA3* (Botstein et al., *Gene* 8:17, 1979), *HIS3* (Struhl et al., *ibid.*) or *POT1* (Kawasaki et al., *ibid.*). Another suitable selectable marker is the CAT gene, which confers chloramphenicol resistance on yeast cells.

Promoters suitable for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255:12073-12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet.* 1:419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al. (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol.* 101:192-201, 1983). In this regard, particularly preferred promoters are the *TPI1* promoter (Kawasaki, U.S. Patent No. 4,599,311, 1986) and the *ADH2-4^C* promoter (Russell et al., *Nature* 304:652-654, 1983; Irani and Kilgore, U.S. Patent

Application Serial No. 07/784,653, which is incorporated herein by reference). The expression units may also include a transcriptional terminator, such as the *TPI1* terminator (Alber and Kawasaki, *ibid.*).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi *Aspergillus* (McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the *ADH3* promoter (McKnight et al., *EMBO J.* 4:2093-2099, 1985) and the *tpiA* promoter. An example of a suitable terminator is the *ADH3* terminator (McKnight et al., *ibid.*, 1985). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal nucleic acid of *Aspergillus*.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75:1929-1933, 1978), Yelton et al. (*Proc. Natl. Acad. Sci. USA* 81:1740-1747, 1984), and Russell (*Nature* 301:167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art. To optimize production of the heterologous proteins in yeast, for example, it is preferred that the host strain carries a mutation, such as the yeast *pep4* mutation (Jones, *Genetics* 85:23-33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), and 293 (ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines. A preferred BHK cell line is the BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314). In addition, a number of other mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC No. CRL 1600), Rat Hep II (ATCC No.

CRL 1548), TCMK (ATCC No. CCL 139), Human lung (ATCC No. CCL 75.1), Human hepatoma (ATCC No. HTB-52), Hep G2 (ATCC No. HB 8065), Mouse liver (ATCC No. CCL 29.1), NCTC 1469 (ATCC No. CCL 9.1), SP2/0-Ag14 (ATCC No. 1581), HIT-T15 (ATCC No. CRL 1777), and RINm 5AHT₂B (Orskov and Nielson, *FEBS* 229(1):175-178, 1988).

Mammalian expression vectors for use in carrying out the present invention should include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., *Cell* 41:521-530, 1985) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1:854-864, 1981). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_J promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA* 81:7041-7045, 1983; Grant et al., *Nuc. Acids Res.* 15:5496, 1987) and a mouse V_H promoter (Loh et al., *Cell* 33:85-93, 1983). A particularly preferred promoter is the major late promoter from Adenovirus 2 (Kaufman and Sharp, *Mol. Cell. Biol.* 2:1304-13199, 1982). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the nucleic acid sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from SV40, adenovirus and/or immunoglobulin genes. Alternatively, within certain embodiments RNA splice sites may be located downstream from the nucleic acid sequence encoding the peptide or protein of interest. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Suitable polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse I

enhancer (Gillies, *Cell* 33:717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs. Suitable vectors can be obtained from commercial sources (e.g., Invitrogen, San Diego, CA; Stratagene, La Jolla, CA).

Cloned nucleic acid sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), or DEAE-dextran mediated transfection (Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, 1987), which are incorporated herein by reference. To identify cells that have stably integrated the cloned nucleic acid, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. Preferred amplifiable selectable markers are the DHFR gene and the neomycin resistance gene. Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate vector at the same time as the CRF₂ receptor sequence, or they may be introduced on the same vector. If on the same vector, the selectable marker and the CRF₂ receptor sequence may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous to add additional nucleic acid, known as "carrier nucleic acid" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the nucleic acid sequence(s) of interest. Drug

selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels. Cells expressing the introduced sequences are selected and screened for production of the protein of interest in the desired form or at the desired level. Cells which satisfy these criteria may then be cloned and scaled up for production.

Preferred prokaryotic host cells for use in carrying out the present invention include *Escherichia coli* (e.g., *E. coli* HB101, *E. coli* DH1, *E. coli* MRC1 and *E. coli* W3110), although *Bacillus*, *Pseudomonas*, and *Streptomyces* and other genera are also useful. Techniques for transforming these hosts and expressing foreign nucleic acid sequences cloned therein are well known in the art (see, e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982; or Sambrook et al., *supra*). Vectors used for expressing cloned nucleic acid sequences in bacterial hosts will generally contain a selectable marker, such as a gene for antibiotic resistance, and a promoter that functions in the host cell. Appropriate promoters include the *trp* (Nichols and Yanofsky, *Meth. Enzymol.* 101:155-164, 1983), *lac* (Casadaban et al., *J. Bacteriol.* 143:971-980, 1980), and phage *k* (Queen, *J. Mol. Appl. Genet.* 2:1-10, 1983) promoter systems. Plasmids useful for transforming bacteria include pBR322 (Bolivar et al., *Gene* 2:95-113, 1977), the pUC plasmids (Messing, *Meth. Enzymol.* 101:20-78, 1983; Vieira and Messing, *Gene* 19:259-268, 1982), pCQV2 (Queen, *ibid.*), pMAL-2 (New England Biolabs, Beverly, MA) and derivatives thereof. Plasmids may contain both viral and bacterial elements.

Given the teachings provided herein, promoters, terminators and methods for introducing expression vectors encoding CRF₂ receptors of the present invention into plant, avian and insect cells would be evident to those of skill in the art. The use of baculoviruses, for example, as vectors for expressing heterologous nucleic acid sequences in insect cells has been reviewed by Atkinson et al. (*Pestic. Sci.* 28:215-

224,1990). In addition, the use of *Agrobacterium rhizogenes* as vectors for expressing genes in plant cells has been reviewed by Sinkar et al. (*J. Biosci. (Bangalore)* 11:47-58, 1987).

Host cells containing nucleic acid molecules of the present invention are then cultured to express a nucleic acid molecule encoding a CRF₂ receptor. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the chosen host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals, as well as other components, e.g., growth factors or serum, that may be required by the particular host cells. The growth medium will generally select for cells containing the nucleic acid molecules by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the nucleic acid construct or co-transfected with the nucleic acid construct.

Suitable growth conditions for yeast cells, for example, include culturing in a chemically defined medium, comprising a nitrogen source, which may be a non-amino acid nitrogen source or a yeast extract, inorganic salts, vitamins and essential amino acid supplements at a temperature between 4°C and 37°C, with 30°C being particularly preferred. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, more preferably pH 5-6. Methods for maintaining a stable pH include buffering and constant pH control. Preferred agents for pH control include sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Due to the tendency of yeast host cells to hyperglycosylate heterologous proteins, it may be preferable to express the CRF₂ receptors of the present invention in yeast cells having a defect in a gene required for asparagine-linked glycosylation. Such cells are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M, preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally cultured in commercially available serum-

containing or serum-free media. Selection of a medium and growth conditions appropriate for the particular cell line used is within the level of ordinary skill in the art.

CRF₂ receptors may also be expressed in non-human transgenic animals, particularly transgenic warm-blooded animals. Methods for producing transgenic animals, including mice, rats, rabbits, sheep and pigs, are known in the art and are disclosed, for example, by Hammer et al. (*Nature* 315:680-683, 1985), Palmiter et al. (*Science* 222:809-814, 1983), Brinster et al. (*Proc. Natl. Acad. Sci. USA* 82:4438-4442, 1985), Palmiter and Brinster (*Cell* 41:343-345, 1985) and U.S. Patent No. 4,736,866, which are incorporated herein by reference. Briefly, an expression unit, including a nucleic acid sequence to be expressed together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs. Introduction of nucleic acid is commonly done by microinjection. Integration of the injected nucleic acid is detected by blot analysis of nucleic acid from tissue samples, typically samples of tail tissue. It is generally preferred that the introduced nucleic acid be incorporated into the germ line of the animal so that it is passed on to the animal's progeny.

Within particularly preferred embodiments of the invention, "knockout" animals may be developed from embryonic stem cells through the use of homologous recombination (Capecchi, *Science* 244:1288-1292, 1989) or antisense oligonucleotide (Stein and Chen, *Science* 261(5124):1004-1012, 1993; Milligan et al., *Semin. Conc. Biol.* 3(6):391-398, 1992).

Within a preferred embodiment of the invention, a transgenic animal, such as a mouse, is developed by targeting a mutation to disrupt a CRF₂ receptor sequence (see Mansour et al., "Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: A general strategy for targeting mutations to non-selectable genes," *Nature* 336:348-352, 1988). Such animals may readily be utilized as a model to study the role of the CRF₂ receptor in metabolism.

CRF₂ Receptor Peptides

As noted above, the present invention also provides CRF₂ receptor peptides. Within the context of the present invention, CRF₂ receptor peptides should be understood to include portions of a CRF₂ receptor or derivatives thereof discussed above, which do not contain transmembrane domains, and which are at least 8, and more preferably 10 or greater amino acids in length. Briefly, the structure of the CRF₂ receptor as well as putative transmembrane domains may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mt. View, CA), or according to the methods described by Kyte and Doolittle (*J. Mol. Biol.* 157:105-132, 1982). While not wishing to be bound by a graphical representation, based upon this hydrophobicity analysis, CRF₂ receptors are believed to have the general structure shown in Figures 1 and 2. In particular, these receptors are believed to comprise an extracellular amino-terminal domain, three extracellular loop domains and four intracellular loop domains each separated by a transmembrane domain.

Within one aspect of the invention, isolated CRF₂ receptor peptides are provided comprising the extracellular amino-terminal domain of a CRF₂ receptor. Within a preferred embodiment, an isolated CRF₂ receptor peptide is provided comprising the sequence of amino acids shown in Sequence I.D. No. 4, from amino acid 1 to amino acid number 118. Within another embodiment, an isolated CRF₂ receptor peptide is provided comprising the sequence of amino acids shown in Sequence ID No. 2 from amino acid number 1 to amino acid number 138.

CRF₂ receptor peptides may be prepared by, among other methods, culturing suitable host/vector systems to produce the recombinant translation products of the present invention. Supernatants from such cell lines may then be treated by a variety of purification procedures in order to isolate the CRF₂ receptor peptide. For example, the supernatant may be first concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following concentration, the concentrate may be applied to a suitable purification matrix

such as, for example, CRF or an anti-CRF₂ receptor antibody bound to a suitable support. Alternatively, anion or cation exchange resins may be employed in order to purify the receptor or peptide. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps may be employed to further purify the CRF₂ receptor peptide.

Alternatively, CRF₂ receptor peptides may also be prepared utilizing standard polypeptide synthesis protocols, and purified utilizing the above-described procedures.

A CRF₂ receptor peptide is deemed to be "isolated" or purified within the context of the present invention, if only a single band is detected subsequent to SDS-polyacrylamide gel analysis followed by staining with Coomassie Brilliant Blue.

Antibodies To CRF₂ Receptors

Within one aspect of the present invention, CRF₂ receptors, including derivatives thereof, as well as portions or fragments of these proteins such as the CRF₂ receptor peptides discussed above, may be utilized to prepare antibodies which specifically bind to CRF₂ receptors. Within the context of the present invention the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, fragments thereof such as F(ab')₂ and Fab fragments, as well as recombinantly produced binding partners. These binding partners incorporate the variable regions from a gene which encodes a specifically binding monoclonal antibody. Antibodies are defined to be specifically binding if they bind to the CRF₂ receptor with a K_a of greater than or equal to 10⁷ M⁻¹. The affinity of a monoclonal antibody or binding partner may be readily determined by one of ordinary skill in the art (see Scatchard, *Ann. N.Y. Acad. Sci.* 51:660-672, 1949).

Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats. Briefly, the CRF₂ receptor is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous

injections. The immunogenicity of a CRF₂ receptor or CRF₂ receptor peptide may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, small samples of serum are collected and tested for reactivity to the CRF₂ receptor. A variety of assays may be utilized in order to detect antibodies which specifically bind to a CRF₂ receptor. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: Countercurrent Immuno-Electrophoresis (CIEP), Radioimmunoassays, Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530; see also *Antibodies: A Laboratory Manual, supra*). Particularly preferred polyclonal antisera will give a signal that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the CRF₂ receptor, larger quantities of polyclonal antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using well-known techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; see also *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Briefly, within one embodiment a subject animal such as a rat or mouse is injected with a form of CRF₂ receptor suitable for generating an immune response against the CRF₂ receptor. Representative examples of suitable forms include, among others, cells which express the CRF₂ receptor, or peptides which are based upon the CRF₂ receptor sequence. Additionally, many techniques are known in the art for increasing the resultant immune response, for example, by coupling the receptor or receptor peptides to another protein such as ovalbumin or keyhole limpet hemocyanin (KLH), or through the use of adjuvants such as Freund's complete or incomplete

adjuvant. The initial immunization may be through intraperitoneal, intramuscular, intraocular, or subcutaneous routes.

Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization. The animal may then be bled and the serum tested for binding to the CRF₂ receptor using assays as described above. Additional immunizations may also be accomplished until the animal has plateaued in its reactivity to the CRF₂ receptor. The animal may then be given a final boost of CRF₂ receptor or CRF₂ receptor peptide, and three to four days later sacrificed. At this time, the spleen and lymph nodes may be harvested and disrupted into a single cell suspension by passing the organs through a mesh screen or by rupturing the spleen or lymph node membranes which encapsidate the cells. Within one embodiment the red cells are subsequently lysed by the addition of a hypotonic solution, followed by immediate return to isotonicity.

Within another embodiment, suitable cells for preparing monoclonal antibodies are obtained through the use of *in vitro* immunization techniques. Briefly, an animal is sacrificed, and the spleen and lymph node cells are removed as described above. A single cell suspension is prepared, and the cells are placed into a culture containing a form of the CRF₂ receptor that is suitable for generating an immune response as described above. Subsequently, the lymphocytes are harvested and fused as described below.

Cells which are obtained through the use of *in vitro* immunization or from an immunized animal as described above may be immortalized by transfection with a virus such as the Epstein-Barr virus (EBV) (see Glasky and Reading, *Hybridoma* 8(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibodies. Suitable myeloma lines are preferably defective in the construction or expression of antibodies, and are additionally syngeneic with the cells from the immunized animal. Many such myeloma cell lines are well known in the art and may be obtained from sources such as

the American Type Culture Collection (ATCC), Rockville, Maryland (see *Catalogue of Cell Lines & Hybridomas*, 6th ed., ATCC, 1988). Representative myeloma lines include: for humans, UC 729-6 (ATCC No. CRL 8061), MC/CAR-Z2 (ATCC No. CRL 8147), and SKO-007 (ATCC No. CRL 8033); for mice, SP2/0-Ag14 (ATCC No. CRL 1581), and P3X63Ag8 (ATCC No. TIB 9); and for rats, Y3-Ag1.2.3 (ATCC No. CRL 1631), and YB2/0 (ATCC No. CRL 1662). Particularly preferred fusion lines include NS-1 (ATCC No. TIB 18) and P3X63 - Ag 8.653 (ATCC No. CRL 1580), which may be utilized for fusions with either mouse, rat, or human cell lines. Fusion between the myeloma cell line and the cells from the immunized animal may be accomplished by a variety of methods, including the use of polyethylene glycol (PEG) (see *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988) or electrofusion (see Zimmerman and Vienken, *J. Membrane Biol.* 67:165-182, 1982).

Following the fusion, the cells are placed into culture plates containing a suitable medium, such as RPMI 1640 or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, KS). The medium may also contain additional ingredients, such as Fetal Bovine Serum ("FBS," *i.e.*, from Hyclone, Logan, Utah, or JRH Biosciences), thymocytes which were harvested from a baby animal of the same species as was used for immunization, or agar to solidify the medium. Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells. Particularly preferred is the use of HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, MO). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which recognize the CRF₂ receptor. Following several clonal dilutions and reassays, a hybridoma producing antibodies which bind to CRF₂ receptor may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (see Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science* 246:1275-1281, December 1989; see also

Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," *Proc. Natl. Acad. Sci. USA* 86:5728-5732, August 1989; see also Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," *Strategies in Molecular Biology* 3:1-9, January 1990; these references describe a commercial system available from Stratagene, La Jolla, California, which enables the production of antibodies through recombinant techniques). Briefly, mRNA is isolated from a B cell population and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the kIMMUNOZAP(H) and kIMMUNOZAP(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al., *supra*; see also Sastry et al., *supra*). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, binding partners may also be constructed utilizing recombinant nucleic acid techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. The construction of these proteins may be readily accomplished by one of ordinary skill in the art (see Larrick et al., "Polymerase Chain Reaction Using Mixed Primers: Cloning of Human Monoclonal Antibody Variable Region Genes From Single Hybridoma Cells," *Biotechnology* 7:934-938, September 1989; Riechmann et al., "Reshaping Human Antibodies for Therapy," *Nature* 332:323-327, 1988; Roberts et al., "Generation of an Antibody with Enhanced Affinity and Specificity for its Antigen by Protein Engineering," *Nature* 328:731-734, 1987; Verhoeven et al., "Reshaping Human Antibodies: Grafting an Antilysozyme Activity," *Science* 239:1534-1536, 1988; Chaudhary et al., "A Recombinant Immunotoxin Consisting of Two Antibody Variable Domains Fused to *Pseudomonas* Exotoxin," *Nature* 339:394-397, 1989; see also, U.S. Patent No. 5,132,405 entitled "Biosynthetic Antibody Binding Sites"), given the disclosure provided herein. Briefly, within one embodiment, nucleic acid molecules encoding CRF₂ receptor-specific antigen binding domains are amplified from hybridomas which produce a specifically binding

monoclonal antibody, and inserted directly into the genome of a cell which produces human antibodies (see Verhoeyen et al., *supra*; see also Reichmann et al., *supra*). This technique allows the antigen-binding site of a specifically binding mouse or rat monoclonal antibody to be transferred into a human antibody. Such antibodies are preferable for therapeutic use in humans because they are not as antigenic as rat or mouse antibodies.

Alternatively, the antigen-binding sites (variable region) may be either linked to, or inserted into, another completely different protein (see Chaudhary et al., *supra*), resulting in a new protein with antigen-binding sites of the antibody as well as the functional activity of the completely different protein. As one of ordinary skill in the art will recognize, the antigen-binding sites or CRF₂ receptor binding domain of the antibody may be found in the variable region of the antibody. Furthermore, nucleic acid sequences which encode smaller portions of the antibody or variable regions which specifically bind to mammalian CRF₂ receptor may also be utilized within the context of the present invention. These portions may be readily tested for binding specificity to the CRF₂ receptor utilizing assays described below.

Within a preferred embodiment, genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using oligonucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Stratacyte (La Jolla, CA) sells primers for mouse and human variable regions including, among others, primers for V_{HA}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions. These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as IMMUNOZAP*(H) or IMMUNOZAP*(L) (Stratacyte), respectively. These vectors may then be introduced into *E. coli* for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (see Bird et al., *Science* 242:423-426, 1988).

Other "antibodies" which may also be prepared utilizing the disclosure provided herein, and thus which are also deemed to fall within the scope of the present invention include humanized antibodies (e.g., U.S. Patent No. 4,816,567 and WO 94/10332), microbodies (e.g., WO 94/09817) and transgenic antibodies (e.g., GB 2 272 440).

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see *Antibodies: A Laboratory Manual, supra*). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques. Within the context of the present invention, the term "isolated" as used to define antibodies or binding partners means "substantially free of other blood components."

Antibodies of the present invention have many uses. For example, antibodies may be utilized in flow cytometry to sort CRF₂ receptor-bearing cells, or to histochemically stain CRF₂ receptor-bearing tissues. Briefly, in order to detect CRF₂ receptors on cells, the cells (or tissue) are incubated with a labeled antibody which specifically binds to CRF₂ receptors, followed by detection of the presence of bound antibody. These steps may also be accomplished with additional steps such as washings to remove unbound antibody. Representative examples of suitable labels, as well as methods for conjugating or coupling antibodies to such labels are described in more detail below.

In addition, purified antibodies may also be utilized therapeutically to block the binding of CRF or other CRF₂ receptor substrates such as sauvagine or urotensin I to the CRF₂ receptor *in vitro* or *in vivo*. Briefly, blocking antibodies are those antibodies that bind to CRF₂ receptor epitopes in such a way as to prevent CRF from binding to the receptor, or to prevent CRF from effecting signal transduction. As noted above, a variety of assays may be utilized to detect antibodies which block or inhibit the binding of CRF to the CRF₂ receptor, including *inter alia*, inhibition and competition assays noted above. Within one embodiment, monoclonal antibodies (prepared as described

above) are assayed for binding to the CRF₂ receptor in the absence of CRF, as well as in the presence of varying concentrations of CRF. Blocking antibodies are identified as those which, for example, bind to CRF₂ receptors and, in the presence of CRF, block or inhibit the binding of CRF to the CRF₂ receptor.

Antibodies of the present invention may also be coupled or conjugated to a variety of other compounds for either diagnostic or therapeutic use. Such compounds include, for example, toxic molecules, molecules which are nontoxic but which become toxic upon exposure to a second compound, and radionuclides. Representative examples of such molecules are described in more detail below.

Antibodies which are to be utilized therapeutically are preferably provided in a therapeutic composition comprising the antibody or binding partner and a physiologically acceptable carrier or diluent. Suitable carriers or diluents include, among others, neutral buffered saline or saline, and may also include additional excipients or stabilizers such as buffers, sugars such as glucose, sucrose, or dextrose, chelating agents such as EDTA, and various preservatives.

Isolation and use of compounds which bind the CRF₂ Receptor, Including Agonists and Antagonists

As noted above, the present invention provides a variety of methods for detecting the presence of compounds which bind to CRF₂ receptors. For example, within one embodiment of the invention methods for detecting such compounds are provided, comprising the steps of (a) exposing one or more compounds to cells that express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compounds to the receptors, and (b) isolating compounds which bind to the receptors, such that the presence of a compound which binds to a CRF₂ receptor may be detected. As utilized herein, conditions sufficient to allow binding of compounds to cells that express CRF₂ receptors generally range from pH 6.8 to 7.5, and include a suitable buffer such as Tris-HCl or PBS, either with or without bovine serum albumin ("BSA"). Such buffers may also include magnesium at a concentration of 5 to 20 mM,

as well as protease inhibitors such as bacitracin or aprotinin. Sufficient time for the binding of compounds to cells that express CRF₂ receptors generally ranges from between 30 and 150 minutes after exposure.

Within other aspects of the present invention, methods for detecting the presence of a compound which binds to CRF₂ receptors are provided, comprising the steps of (a) exposing one or more compounds to a CRF₂ receptor N-terminal extracellular domain under conditions and for a time sufficient to allow binding of a compound to the N-terminal extracellular domain, and (b) isolating compounds which bind to the CRF₂ receptor N-terminal extracellular domain, such that the presence of a compound which binds to a CRF₂ receptor may be detected. Such assays may take a variety of forms, including for example, as Enzyme Linked ImmunoSorbent Assay, "Sandwich" assay, or the like. Within one embodiment, the compounds are labeled with an agent selected from the group consisting of fluorescent molecules, enzymes, and radionuclides.

In addition to providing assays which detect the presence of compounds which bind to CRF₂ receptors, the present invention also provides methods for detecting both CRF₂ receptor agonists and CRF₂ receptor antagonists. Within the context of the present invention, CRF₂ receptor agonists should be understood to refer to molecules that are capable of binding to the cell-surface receptor, thereby stimulating a response pathway within the cell. In contrast, CRF₂ receptor antagonists should be understood to refer to molecules that are capable of binding to a CRF₂ receptor, but which prevent stimulation, or exhibit greatly reduced stimulation of a response pathway within the cell. Various assays may be utilized given the disclosure provided herein in order to screen or select for CRF agonists and antagonists.

For example, within one aspect of the present invention, methods are provided for determining whether a selected compound is a CRF₂ receptor agonist or antagonist, comprising the steps of (a) exposing a selected compound to cells which express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compound and an associated response in intracellular levels of cAMP, and (b)

detecting either an increase or decrease in the level of intracellular cAMP, and thereby determining whether the selected compound is a CRF₂ receptor agonist or antagonist.

cAMP may be readily measured using methods which are well known in the art, including, for example, methods described by Salomon et al. (*Anal. Biochem.* 58:541-548, 1976) or Krishna et al. (*J. Pharmacol. Exp. Ther.* 163:379, 1968), or, preferably, using commercially available kits such as the Scintillation Proximity Assay Kit from Amersham Corporation, or the ¹²⁵I RIA cAMP determination kit from Incstar Corporation (Stillwater, Minnesota). The Scintillation Proximity Assay Kit measures the production of cAMP by competition of iodinated-cAMP with anti-cAMP antibodies.

Within other aspects, methods are provided for detecting the presence of a CRF₂ receptor agonist or antagonist in a pool of compounds, comprising the steps of (a) exposing a pool of compounds to cells which express CRF₂ receptors under conditions and for a time sufficient to allow binding of the compound and an associated response in intracellular levels of cAMP, and (b) isolating compounds which either increase or decrease the intracellular level of cAMP, such that the presence of a CRF₂ receptor agonist or antagonist may be detected.

Within another aspect, methods for determining whether a selected compound is a CRF₂ receptor antagonist are provided, comprising the steps of (a) exposing a selected compound in the presence of a CRF₂ receptor agonist to a recombinant CRF₂ receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the compound to the receptor and an associated response through the pathway, and (b) detecting a reduction in the stimulation of the response pathway resulting from the binding of the compound to the CRF₂ receptor, relative to the stimulation of the response pathway by the CRF₂ receptor agonist alone, and therefrom determining the presence of a CRF₂ antagonist. Within other aspects, methods are provided for determining whether a selected compound is a CRF₂ receptor agonist, comprising the steps of (a) exposing a selected compound to a recombinant CRF₂ receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the compound to the receptor and an associated response

through the pathway, and (b) detecting an increase in stimulation of the response pathway resulting from the binding of the compound to the CRF₂ receptor, and therefrom determining the presence of a CRF₂ receptor agonist.

Within particularly preferred embodiments of the invention, the response pathway is a membrane-bound adenylate cyclase response pathway, and the step of detecting comprises measuring the increase or decrease in cAMP production by the membrane-bound adenylate cyclase response pathway. Adenylate cyclase activity assays may be carried out, for example, utilizing method(s) described below in the Examples. Generally, such methods measure the level of stimulation of cAMP relative to known agonists (e.g., sauvagine, urotensin I or CRF), and generally involve exposing a preparation of cells which express a biologically active CRF₂ receptor to the selected compound in the presence of radiolabelled ATP.

Within a further embodiment of the invention, the response pathway includes a reporter system, such as luciferase, or β -galactosidase (see Konig et al., *Mol. and Cell. Neuro.* 2:331-337, 1991). For examples, within one embodiment of the invention an expression vector is provided comprising a cyclic AMP response element such as a proenkephalin cyclic AMP response element, operably linked to β -galactosidase or luciferase cDNA. The expression vector is then stably transfected into a host cell, and the host cell then transfected with a second expression vector which expresses a CRF₂ receptor. Upon activation of the response pathway, elevated cAMP levels induce the expression of the reporter product, such as the β -galactosidase or luciferase. If the reporter product is luciferase, it is then exposed to luciferin, and photons which are released during the oxidation of luciferin by the luciferase are measured.

A variety of compounds may be screened utilizing such methods. Representative examples include blocking antibodies discussed above, CRF₂ receptor peptides, and CRF analogs (including both peptide and non-peptide ligands). In addition, large numbers of CRF analogs may be generated by saturation mutagenesis of nucleic acid sequences encoding CRF (e.g., Little, *Gene* 88:113-115, 1990; Hembers

et al., *Gene* 88:143-151, 1989), by segment-directed mutagenesis (e.g., Shortle et al., *Proc. Natl. Acad. Sci. USA* 77:5375-5379, 1980), by forced nucleotide misincorporation (e.g., Liao and Wise, *Gene* 88:107-111, 1990), or by use of randomly mutagenized oligonucleotides (Hutchison et al., *Proc. Natl. Acad. Sci. USA* 83:710-714, 1986). Individual transformants expressing a CRF₂ analog may then be cloned as discussed above, or pooled.

Other compounds which may also be screened utilizing methods of the present invention, include chemical compounds which are known and either available commercially (e.g., from Sigma Chemical Co., St. Louis, MO) or readily synthesized by one of skill in the art. In addition, numerous novel compositions which are provided in combinatorial libraries may also be readily screened utilizing the methods described herein, including for example organic and protein or peptide libraries (see e.g., Gold and Tuerk, U.S. Patent No. 5,270,163; and Ladner et al., U.S. Patent Nos. 5,096,815, 5,198,346, and 5,223,409).

Within a preferred aspect of the invention, the screening of chemical libraries (including peptide, small organic molecule or combinatorial chemistry-derived compound libraries) can be assessed in a high-throughput format using the expressed CRF₂ receptors. More specifically, within one embodiment of the invention, a high throughput receptor binding assay may be performed in a 96-well plate and is automated using the BIOMEK 2000 (Beckman, Fullerton CA). Briefly, 1×10^5 cells transfected with the CRF₂ receptor are grown in each well. To this, 0.05 ml of assay buffer (Dulbecco's Phosphate Buffered Saline, 10 mM MgCl₂, 20 μ M Bacitracin) with or without unlabeled r/hCRF (final concentration, 1 μ M) is added in duplicate to determine total binding and non-specific binding. In singlets, 0.05 ml of compounds (mixtures from 1 to 30 compounds) are added to each well in addition to 0.05 ml of either [¹²⁵I]-oCRF or [¹²⁵I]-r/hCRF (final concentration ~200 pM). The mixture is incubated for 2 hours at 22°C. Since the transfected cells are adherent, a centrifugation step to separate membrane-bound CRF is not necessary. Next, the fluid is aspirated and the cells are washed three times with approximately 0.9 ml of PBS. After the third wash

and aspiration, 0.2 ml of 4M guanidine thiocyanate is added to each well to solubilize the tissue. An aliquot (0.15 ml) of the solubilized sample is monitored in a gamma counter for radioactivity at approximately 80% efficiency. Compounds which demonstrate $\geq 50\%$ inhibition of [^{125}I]CRF binding are tested in a full dose response competition assay to determine the affinity of these compounds (K_i value) at the CRF₂ receptor.

Once purified partially, or to homogeneity, as desired, both CRF₂ receptor agonists and antagonists may be used therapeutically. Generally substantially pure recombinant CRF antagonists of at least about 50% homogeneity are preferred, at least about 70%-80% homogeneity more preferred, and 95%-99% or more homogeneity most preferred, particularly for pharmaceutical uses. In general, the antagonists may be administered intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or directly into a tumor.. Typically, the antagonists are present as free bases or as acid salts. Suitable salts should be pharmaceutically acceptable. Representative examples include metal salts, alkali and alkaline earth metal salts such as potassium or sodium salts. Other pharmaceutically acceptable salts include citric, succinic, lactic, hydrochloric and hydrobromic acids. Parenteral compositions may be formulated in aqueous isotonic solutions of between pH 5.6 and 7.4. Suitable isotonic solutions may include sodium chloride, dextrose, boric acid sodium tartrate, and propylene glycol solutions.

Labels

The nucleic acid molecules, antibodies, CRF₂ receptors, and CRF₂ receptor agonists and antagonists of the present invention may be labeled or conjugated (either through covalent or non-covalent means) to a variety of labels or other molecules, including for example, fluorescent markers, enzyme markers, toxic molecules, molecules which are nontoxic but which become toxic upon exposure to a second compound, and radionuclides.

Representative examples of fluorescent labels suitable for use within the present invention include, for example, Fluorescein Isothiocyanate (FITC), Rodamine, Texas Red, Luciferase and Phycoerythrin (PE). Particularly preferred for use in flow cytometry is FITC which may be conjugated to purified antibody according to the method of Keltkamp in "Conjugation of Fluorescein Isothiocyanate to Antibodies. I. Experiments on the Conditions of Conjugation," *Immunology* 18:865-873, 1970. (See also Keltkamp, "Conjugation of Fluorescein Isothiocyanate to Antibodies. II. A Reproducible Method," *Immunology* 18:875-881, 1970; and Goding, "Conjugation of Antibodies with Fluorochromes: Modification to the Standard Methods," *J. Immunol. Methods* 13:215-226, 1970.) For histochemical staining, HRP, which is preferred, may be conjugated to the purified antibody according to the method of Nakane and Kawaoi ("Peroxidase-Labeled Antibody: A New Method of Conjugation," *J. Histochem. Cytochem.* 22:1084-1091, 1974; see also, Tijssen and Kurstak, "Highly Efficient and Simple Methods for Preparation of Peroxidase and Active Peroxidase Antibody Conjugates for Enzyme Immunoassays," *Anal. Biochem.* 136:451-457, 1984).

Representative examples of enzyme markers or labels include alkaline phosphatase, horse radish peroxidase, and β -galactosidase. Representative examples of toxic molecules include ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Representative examples of molecules which are nontoxic, but which become toxic upon exposure to a second compound include thymidine kinases such as HSVTK and VZVTk. Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212.

As will be evident to one of skill in the art given the disclosure provided herein, the above described nucleic acid molecules, antibodies, CRF₂ receptors, CRF₂ receptor peptides and CRF₂ receptor agonists and antagonists may also be labeled with other molecules such as colloidal gold, as well either member of a high affinity binding pair (e.g., avidin-biotin).

Diagnostic Use Of CRF₂ Receptor Sequences

Within another aspect of the present invention, probes and primers are provided for detecting CRF₂ receptors. Within one embodiment of the invention, probes are provided which are capable of hybridizing to CRF₂ receptor nucleic acid or RNA. For purposes of the present invention, probes are "capable of hybridizing" to CRF₂ receptor nucleic acid if they hybridize to Sequence I.D. Nos. 1 or 3 under conditions of moderate or high stringency (see Sambrook et al., *supra*); but not to CRF receptor nucleic acids. Preferably, the probe may be utilized to hybridize to suitable nucleotide sequences in the presence of 50% formamide, 5x SSPE, 5x Denhardt's, 0.1% SDS and 100 ug/ml Salmon Sperm nucleic acid at 42°C, followed by a first wash with 2x SSC at 42°C, and a second wash with 0.2x SSC at 55 to 60°C.

Probes of the present invention may be composed of either deoxyribonucleic acids (DNA) ribonucleic acids (RNA), nucleic acid analogues, or any combination of these, and may be as few as about 12 nucleotides in length, usually about 14 to 18 nucleotides in length, and possibly as large as the entire sequence of the CRF₂ receptor. Selection of probe size is somewhat dependent upon the use of the probe. For example, in order to determine the presence of various polymorphic forms of the CRF₂ receptor within an individual, a probe comprising virtually the entire length of the CRF₂ receptor coding sequence is preferred. CRF₂ receptor probes may be utilized to identify polymorphisms linked to the CRF₂ receptor gene (*see, for example*, Weber, *Genomics* 7:524-530, 1990; and Weber and May, *Amer. J. Hum. Gen.* 44:388-396, 1989). Such polymorphisms may be associated with inherited diseases such as diabetes.

Probes may be constructed and labeled using techniques which are well known in the art. Shorter probes of, for example, 12 or 14 bases may be generated synthetically. Longer probes of about 75 bases to less than 1.5 kb are preferably generated by, for example, PCR amplification in the presence of labeled precursors such as ³²P-dCTP, digoxigenin-dUTP, or biotin-dATP. Probes of more than 1.5 kb are generally most easily amplified by transfecting a cell with a plasmid containing the

relevant probe, growing the transfected cell into large quantities, and purifying the relevant sequence from the transfected cells (see Sambrook et al., *supra*).

Probes may be labeled by a variety of markers, including, for example, radioactive markers, fluorescent markers, enzymatic markers, and chromogenic markers. The use of ^{32}P is particularly preferred for marking or labeling a particular probe.

Probes of the present invention may also be utilized to detect the presence of a CRF₂ receptor mRNA or nucleic acid within a sample. However, if CRF₂ receptors are present in only a limited number, or if it is desired to detect a selected mutant sequence which is present in only a limited number, or if it is desired to clone a CRF₂ receptor from a selected warm-blooded animal, then it may be beneficial to amplify the relevant sequence such that it may be more readily detected or obtained.

A variety of methods may be utilized in order to amplify a selected sequence, including, for example, RNA amplification (see Lizardi et al., *Bio/Technology* 6:1197-1202, 1988; Kramer et al., *Nature* 339:401-402, 1989; Lomeli et al., *Clinical Chem.* 35(9):1826-1831, 1989; U.S. Patent No. 4,786,600), and nucleic acid amplification utilizing Polymerase Chain Reaction ("PCR") (see U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159) (see *also*, U.S. Patent Nos. 4,876,187, and 5,011,769, which describe an alternative detection/amplification system comprising the use of scissile linkages).

Within a particularly preferred embodiment, PCR amplification is utilized to detect or obtain a CRF₂ receptor nucleic acid. Briefly, as described in greater detail below, a nucleic acid sample is denatured at 95°C in order to generate single stranded nucleic acid. Specific primers, as discussed below, are then annealed at 37°C to 70°C, depending on the proportion of AT/GC in the primers. The primers are extended at 72°C with Taq polymerase in order to generate the opposite strand to the template. These steps constitute one cycle, which may be repeated in order to amplify the selected sequence.

Primers for the amplification of a selected sequence should be selected from sequences which are highly specific and form stable duplexes with the target sequence. The primers should also be non-complementary, especially at the 3' end, should not form dimers with themselves or other primers, and should not form secondary structures or duplexes with other regions of nucleic acid. In general, primers of about 18 to 20 nucleotides are preferred, and may be easily synthesized using techniques well known in the art.

Therapeutic Uses of CRF₂ Receptor(s) and CRF₂ Receptor Antagonists

CRF₂ receptors (or portions thereof), CRF₂ receptor agonists and antagonists, as well as the nucleic acid sequences which encode these molecules may be utilized in a variety of therapeutic applications. For example, within one embodiment of the invention CRF₂ receptors (or portions thereof which bind a substrate such as CRF, sauvagine, or urotensin I) may be utilized in order to reduce vascular levels of the substrate or high ACTH levels due to an excess of the substrate. Thus, CRF₂ receptors may be utilized in treating diseases which are associated with high cortisol levels, including for example Cushing's Disease, alcoholism, anorexia nervosa, and other related disorders.

Similarly, CRF₂ receptors, (or portions thereof which bind a substrate as discussed above) may be utilized in treating tumors which produce high levels of a substrate (e.g., CRF) such as pituitary tumors, as well as for treating abnormalities during pregnancy such as preclampsia which are associated with increased CRF levels. Similarly, they may be utilized to treat hypotension, to modulate the effect of immune system disorders such as arthritis, and to modulate the effect of pituitary disorders. In addition, CRF₂ receptors, (or portions thereof) may be utilized in order to modulate a variety of brain functions, including for example control of satiety, reproduction growth, anxiety, depression, fever, metabolism.

Within yet another aspect of the present invention, viral vectors are provided which may be utilized to treat diseases wherein either the CRF₂ receptor (or a

mutant CRF₂ receptor) is over-expressed, or where no CRF₂ receptor is expressed. Briefly, within one embodiment of the invention, viral vectors are provided which direct the production of antisense CRF₂ receptor RNA, in order to prohibit the over-expression of CRF₂ receptors, or the expression of mutant CRF₂ receptors. Within another embodiment, viral vectors are provided which direct the expression of CRF₂ receptor cDNA. Viral vectors suitable for use in the present invention include, among others, herpes viral vectors (e.g., U.S. Patent No. 5,288,641), retroviruses (e.g., EP 0,415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res.* 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram et al., *Cancer Res.* 53:83-88, 1993; Takamiya et al., *J. Neurosci. Res* 33:493-503, 1992; Baba et al., *J. Neurosurg* 79:729-735, 1993), pseudotyped viruses, adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Kolls et al., *PNAS* 91(1):215-219, 1994; Kass-Eisler et al., *PNAS* 90(24):11498-502, 1993; Guzman et al., *Circulation* 88(6):2838-48, 1993; Guzman et al., *Cir. Res.* 73(6):1202-1207, 1993; Zabner et al., *Cell* 75(2):207-216, 1993; Li et al., *Hum Gene Ther.* 4(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci.* 5(10):1287-1291, 1993; Vincent et al., *Nat. Genet.* 5(2):130-134, 1993; Jaffe et al., *Nat. Genet.* 1(5):372-378, 1992; and Levrero et al., *Gene* 101(2):195-202, 1991), adenovirus-associated viral vectors (Flotte et al., *PNAS* 90(22):10613-10617, 1993), parvovirus vectors (Koering et al., *Hum. Gene Therap.* 5:457-463, 1994), baculovirus vectors, and pox virus vectors (Panicali and Paoletti, *PNAS* 79:4927-4931, 1982; and Ozaki et al., *Biochem. Biophys. Res. Comm.* 193(2):653-660, 1993). Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

Within other embodiments of the invention, the vectors which contain or express nucleic acid molecules of the present invention, or even the nucleic acid molecules themselves, may be administered by a variety of alternative techniques, including for example direct nucleic acid injection (Acsadi et al., *Nature* 352:815-818, 1991); liposomes (Pickering et al., *Circ.* 89(1):13-21, 1994; and Wang et al., *PNAS*

84:7851-7855, 1987); lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); nucleic acid ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acid linked to killed adenovirus (Michael et al., *J. Biol. Chem.* 268(10):6866-6869, 1993; and Curiel et al., *Hum. Gene Ther.* 3(2):147-154, 1992), retrotransposons, cytofectin-mediated introduction (DMRIE-DOPE, Vical, Calif.) and transferrin-nucleic acid complexes (Zenke).

Improving Learning and Memory

As noted above, the present invention also provides methods for improving learning and memory by administration to a patient of a therapeutically effective amount of a CRF₂ receptor antagonist. Briefly, such patients may be identified through a clinical diagnosis based on symptoms of dementia or learning and memory loss. For example, individuals with an amnesic disorder are impaired in their ability to learn new information or are unable to recall previously learned information or past events. The memory deficit is most apparent on tasks to require spontaneous recall and may also be evident when the examiner provides stimuli for the person to recall at a later time. The memory disturbance must be sufficiently severe to cause marked impairment in social or occupational functioning and must represent a significant decline from a previous level of functioning.

Dementia is characterized by multiple clinically significant deficits in cognition that represent a significant change from a previous level of functioning. Memory impairment involving inability to learn new material or forgetting of previously learned material is required to make the diagnosis of a dementia. Memory can be formally tested by asking the person to register, retain, recall and recognize information. The diagnosis of dementia also requires at least one of the following cognitive disturbances: aphasia, apraxia, agnosia or a disturbance in executive functioning. These deficits in language, motor performance, object recognition and abstract thinking, respectively, must be sufficiently severe in conjunction with the memory deficit to cause

impairment in occupational or social functioning and must represent a decline from a previously higher level of functioning.

A standard test used by clinicians to determine if a patient has impaired learning and memory is the Minimental Test for Learning and Memory (Folstein et al., *J. Psychiatric Res.* 12:185, 1975). This test involves a number of simple tasks and written questions. For instance, "paired-associate" learning ability is impaired in amnesiac patients of several types including those suffering from head trauma, Korsakoff's disease or stroke (Squire, 1987). Ten pairs of unrelated words (e.g., army-table) are read to the subject. Subjects are then asked to recall the second word when given the first word of each pair. The measure of memory impairment is a reduced number of paired-associate words recalled relative to a matched control group. This serves as an index of short-term, working memory of the kind that deteriorates rapidly in the early stages of dementing or amnesiac disorders.

Improvement in learning and memory constitutes either (a) a statistically significant difference between the performance of CRF₂ receptor antagonist treated patients as compared to members of a placebo group; or (b) a statistically significant change in performance in the direction of normality on measures pertinent to the disease model. Animal models or clinical instances of disease exhibit symptoms which are by definition distinguishable from normal controls. Thus, the measure of effective pharmacotherapy will be a significant, but not necessarily complete, reversal of symptoms. Improvement can be facilitated in both animal and human models of memory pathology by clinically effective "cognitive enhancing" drugs which serve to improve performance of a memory task. For example, cognitive enhancers which function as cholinomimetic replacement therapies in patients suffering from dementia and memory loss of the Alzheimer's type significantly improve short-term working memory in such paradigms as the paired-associate task (Davidson and Stern, 1991). Another potential application for therapeutic interventions against memory impairment is suggested by age-related deficits in performance which are effectively modeled by the longitudinal study of recent memory in aging mice (Forster and Lal, 1992).

In animals, several established models of learning and memory are available to examine the beneficial cognitive enhancing effects and potential anxiety-related side effects of activation of CRF-sensitive neurons. For example, both the Morris maze (Stewart and Morris, in *Behavioral Neuroscience*, R. Saghal, Ed. (IRL Press, 1993) p. 107) and the Y-maze (Brits et al., *Brain Res. Bull.* 6, 71 (1981)) tests measure cognitive enhancing effects. Anxiety-related effects may be evaluated in the elevated plus-maze. (Pellow et al., *J. Neurosci. Meth.* 14:149, 1985.)

Briefly, the Morris water maze is one of the best validated models of learning and memory, and it is sensitive to the cognitive enhancing effects of a variety of pharmacological agents (McNamara and Skelton, *Brain Res. Rev.* 18:33, 1993). The task performed in the maze is particularly sensitive to manipulations of the hippocampus in the brain, an area of the brain important for spatial learning in animals and memory consolidation in humans. Moreover, improvement in Morris water maze performance is predictive of clinical efficacy of a compound as a cognitive enhancer. For example, treatment with cholinesterase inhibitors or selective muscarinic cholinergic agonists reverse learning deficits in the Morris maze animal model of learning and memory, as well as in clinical populations with dementia (McNamara and Skelton, 1993; Davidson and Stern, 1991; McEntee and Crook, 1992; Dawson et al., 1992). In addition, this animal paradigm accurately models the increasing degree of impairment with advancing age (Levy et al., 1994) and the increased vulnerability of the memory trace to pre-test delay or interference (Stewart and Morris, 1993) which is characteristic of amnesiac patients.

The test is a simple spatial learning task in which the animal is placed in tepid water, which is opaque due to the addition of powdered milk. The animals learn the location of the platform relative to visual cues located within the maze and the testing room; this learning is referred to as place learning. Briefly, 15 minutes prior to training on each of days 1-3, groups of animals receive ICV injections of control solution or 0.1, 1, 5, or 25 μ g of a CRF₂ receptor antagonist. Control animals typically reach the

platform within five to ten seconds after three days of training. The measure of the memory modulator effects of a CRF₂ receptor antagonist is a shift of this time period.

The Y-maze test based on visual discrimination is another assay of learning and memory in animals. In this maze, two arms of the maze end in a translucent plastic panel behind which there is a 40-watt electric bulb. The start box is separated from the third arm by a manually-activated guillotine door. In the first trial, all animals are allowed to explore the maze for five minutes, and food pellets are available in each arm. On the second day, each animal is placed in the start box with the door closed. When the door is opened, the animal is allowed to move down the arms and eat the pellets which are located in both arms. On the third day, animals receive six trials in groups of three where one arm is closed at the choice point, no discriminative stimulus is present, and two food pellets are available in the open goal box. On days 4-10, a light at the end of the arm with the food pellets is illuminated and ten trials are run, again in groups of three. The time it takes for the animal to reach the food pellets is recorded.

The effectiveness of a CRF₂ receptor antagonist to improve learning and memory in the Y-maze is tested as follows. Fifteen minutes prior to each of the blocks of training trials on days 4-10, groups of animals receive ICV injections of control solutions or doses of 1, 5, or 25 µg of a CRF₂ receptor antagonist. Control animals are expected to make 50% correct choices. The measure of efficacy of treatment on memory is an increase in correct responses.

The elevated plus maze test measures anxiogenic responses in an approach-avoidance situation involving an exposed, lighted space versus a dark, enclosed space. Both spaces are elevated and are set up as two runways intersecting in the form of a plus sign. This type of approach-avoidance situation is a classical test of "emotionality" and is very sensitive to treatments that produce disinhibition and stress. Briefly, animals are placed in the center of the maze and are allowed free access to all four arms in a five minute testing period. The time spent in each arm is recorded.

In humans, determination of improving learning and memory may be measured by such tests as the Wechsler Memory Scale or a pair-associate memory task. The Wechsler Memory Scale is a widely-used pencil-and-paper test of cognitive function and memory capacity. In the normal population, the standardized test yields a mean of 100 and a standard deviation of 15, so that a mild amnesia can be detected with a 10-15 point reduction in the score, a more severe amnesia with a 20-30 point reduction, and so forth (Squire, 1987). During the clinical interview, a battery of tests, including, but not limited to, the Minimental test, the Wechsler memory scale, or paired-associate learning are applied to diagnose symptomatic memory loss. These tests provide general sensitivity to both general cognitive impairment and specific loss of learning/memory capacity (Squire, 1987). Apart from the specific diagnosis of dementia or amnesic disorders, these clinical instruments also identify age-related cognitive decline which reflects an objective diminution in mental function consequent to the aging process that is within normal limits given the person's age (DSM IV, 1994). As noted above, "improvement" in learning and memory is present within the context of the present invention if there is a statistically significant difference in the direction of normality in the paired-associate test, for example, between the performance of CRF₂ receptor antagonist treated patients as compared to members of the placebo group or between subsequent tests given to the same patient.

Alzheimer's Disease

The present invention provides methods for treating Alzheimer's disease (AD) by administration to a patient of a therapeutically effective amount of a CRF₂ receptor antagonist. Briefly, such patients may be identified through clinical diagnosis based on symptoms of dementia or learning and memory loss which are not attributable to other causes. In addition, patients are also identified through diagnosis of brain atrophy as determined by magnetic resonance imaging.

Several established animal models of Alzheimer's disease which focus on cholinergic deficits are available. The primary role of cholinergic deficits in AD is well

established. In AD, there are significant positive correlations between reduced choline acetyltransferase activity and reduced CRF levels in the frontal, occipital, and temporal lobes (DeSouza et al., 1986). Similarly, there are negative correlations between decreased choline acetyltransferase activity and an increased number of CRF receptors in these three cortices (*Id.*). In two other neurodegenerative diseases, there are highly significant correlations between CRF and choline acetyltransferase activity in Parkinson's disease, but only a slight correlation in progressive supranuclear palsy (Whitehouse et al., 1987).

In rats, anatomic and behavioral studies evidence interactions between CRF and cholinergic systems. First, in some brain stem nuclei, CRF and acetylcholinesterase are co-localized, and some cholinergic neurons also contain CRF. Second, CRF inhibits carbachol-induced behaviors (carbachol is a muscarinic cholinergic receptor antagonist), suggesting that CRF has effects on cholinergic systems (Crawley et al., *Peptides* 6:891, 1985). Treatment with another muscarinic cholinergic receptor antagonist, atropine, results in an increase in CRF receptors (DeSouza and Battaglia, *Brain Res.* 397:401, 1986). Taken together, these data show that CRF and cholinergic systems interact similarly in humans and animals.

An animal model of Alzheimer's disease which focuses on cholinergic deficits is produced by the administration of scopolamine, a non-selective postsynaptic muscarinic receptor antagonist that blocks the stimulation of postsynaptic receptors by acetylcholine. In these animals, memory deficits are readily apparent as measured by passive avoidance or delayed-matching-to-position tests, which distinguish motor or perceptual deficits from amnesia or cognitive enhancing effects of experimental treatments. Thus, the Morris maze and Y-maze tests following scopolamine-induced amnesia are utilized to test memory impairment and subsequent enhancement following administration of a CRF₂ receptor antagonist. In the Morris maze, the design of the experiment is essentially as described above, but is modified to include treatment 30 minutes prior to training on each of days 1 to 3 with an ip injection of scopolamine hydrobromide (0.3 mg/kg). This amnestic dose of scopolamine impairs acquisition and

retention of spatial and avoidance learning paradigms in the rat. The anti-amnestic effects of 1, 5, or 25 μg of a CRF_2 receptor antagonist are measured relative to the concurrent control groups who receive or do not receive scopolamine. The effect of the CRF_2 receptor antagonists on reversal of scopolamine-induced amnesia using the Y-maze is performed similarly to the Y-maze test described above. Modification of this test includes treatment 30 minutes prior to training on days 5 to 10 with an ip injection of scopolamine hydrobromide (0.3 mg/kg). The anti-amnestic effects of 1, 5, or 25 μg of a CRF_2 receptor antagonist administered ICV, centrally or systemically, are measured relative to concurrent control and scopolamine treated-control groups.

Several tests measuring cognitive behavior in AD have also been designed. (See Gershon et al., *Clinical Evaluation of Psychotropic Drugs: Principles and Guidelines*, Prien and Robinson (eds.), Raven Press, Ltd., New York, 1994, p. 467.) One of these tests, BCRS, measures concentration, recent memory, past memory, orientation, and functioning and self-care. The BCRS is designed to measure only cognitive functions. This test, as well as the Weschler Memory Scale and the Alzheimer's Disease-Associated Scale, may be used to determine improvement following therapeutic treatment with a CRF_2 receptor antagonist. As noted above, "improvement" in Alzheimer's disease is present within the context of the present invention if there is a statistically significant difference in the direction of normality in the Weschler Memory Scale test, for example, between the performance of CRF_2 receptor antagonist treated patients as compared to members of the placebo group or between subsequent tests given to the same patient. In addition, scopolamine-induced amnesia in humans can be used as a model system to test the efficacy of the CRF_2 receptor antagonists.

Cerebrovascular Disease

As noted above, the present invention also provides methods for treating cerebrovascular diseases such as stroke, reperfusion injury and migraines, by administration to a patient of a therapeutically effective amount of a CRF_2 receptor

antagonist. A patient is deemed to have been treated if the administration of the CRF₂ receptor antagonist, results in a statistically significant benefit, as compared to controls, of a clinical or diagnostic indication of a cerebrovascular disease (see, e.g., Harrison's Principles of Internal Medicine, McGraw-Hill Book Co.). Representative examples of animal model systems to test the effects of a CRF₂ receptor antagonist upon cerebrovascular disease include focal ischemic damage from injections of NMDA agonists (1-amino cyclobutane-cis-1,3 dicarboxylic acid). Such excitotoxic compounds may be fused into specific areas of the brain, and the toxicity measured by later staining with a compound such as tetrazolium. Indicia of neural protection may be evidenced by the reduction of the volume of infarction in animals treated with a CRF₂ receptor antagonist.

Administration of CRF₂ receptor antagonists may be by a variety of routes, including for example by direct injection into a site of injury or disease, via other intracranial, intradermal, intraperitoneal, intrathecal, subcutaneous, or intramuscular routes, or more preferably, orally or intravenously.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

ISOLATION OF CRF₂ RECEPTOR cDNA

A. Isolation of CRF₂ receptor cDNA from a rat brain cDNA library

Male Sprague-Dawley rats (Madison, WI) weighing between 175-250 gm are decapitated, and the brain excised. Total RNA is then isolated from the brain utilizing a Promega RNAGents Total RNA Kit (catalog #Z5110, Promega, Wisc.) according to the manufacturers instructions, followed by the isolation of poly A+ RNA utilizing a Promega PolyATract kit (catalog # Z5420). A cDNA phage library is then prepared utilizing a Giga-Pack Gold library construction kit according to the manufacturers instructions (catalog #237611, Stratagene, LaJolla, Calif.), which is in turn plated and screened essentially as described by Sambrook et al., (Molecular Cloning) with oligonucleotide 5'-CCCGGATGCC TACAGAGAAT GCCTGGAGGA TGGGACCTGG GCCTCAAGGG-3'' (Sequence I.D. No. 5). This oligonucleotide is complementary to nucleotides 440-490 of the rat CRF₂ receptor cDNA sequence shown in Figure 1.

The phage library is rescreened until a single pure phage isolate is obtained. The phage is then grown on bacterial host XL1-Blue (Stratagene, LaJolla, Calif.), and plasmid nucleic acid is excised with ExAssist helper phage (Stratagene) in SOLR cells. The SOLR cells are then plated, and plasmid nucleic acid is isolated and sequenced utilizing the Sanger dideoxy protocol.

A rat CRF₂ receptor cDNA sequence that may be obtained utilizing this procedure is set forth below in Figure 2 and in Sequence I.D. No. 1.

B. Isolation of CRF₂ receptor cDNA from a rat hypothalamus cDNA library

CRF₂ receptor cDNA can also be isolated from commercially available rat hypothalamus cDNA libraries. Briefly, two million plaques from a rat hypothalamus

phage library (Stratagene, catalog # 936518) are plated according to the manufacturers instructions, and screened with oligonucleotide Sequence I.D. No. 5 essentially as described above.

A rat CRF₂ receptor cDNA sequence that may be obtained utilizing this procedure is set forth below in Figure 1 and in Sequence I.D. No. 3.

C. Isolation of CRF₂ receptor cDNA from a human cDNA library

CRF₂ receptor cDNA can also be isolated from commercially available human cDNA libraries. Briefly, approximately two million plaques from a human frontal cortex phage library (Stratagene, catalog # 936212) are plated according to the manufacturers instructions, and screened with oligonucleotide 5' -GATCAACTAC TCACAGTGTG AGCCCATTTT GGATGACAAG CAGAGGAAGT A-3' (Sequence I.D. No. 6) essentially as described above.

One human CRF₂ receptor cDNA sequence that may be obtained utilizing this procedure is set forth below in Sequence I.D. No. 7. Briefly, the human sequence is 89.1% identical at the nucleotide level and 93.0% identical at the amino acid level to that of the above-described rat CRF_{2α} receptor (Sequence I.D. No. 3).

EXAMPLE 2

EXPRESSION OF CRF₂ RECEPTOR cDNA

pCDM7-Amp is first constructed from pCDM8 (Seed, *Nature* 329:840-842, 1987; Seed and Aruffo, *Proc. Natl. Acad. Sci.* 84:3365-3369, 1987; Thomsen et al., *Cell* 63:485-493, 1990; Bernot and Auffray, *Proc. Natl. Acad. Sci.* 88:2550-2554, 1991; Han et al., *Nature* 349:697-700, 1991) by deletion of the adeno origin of replication, M13 origin of replication and sup F selection marker. An ampicillin resistance marker is added in order to facilitate selection of the plasmid.

A full-length CRF₂ receptor clone in pBluescriptSK- is isolated from the phage clone described above, and cut with EcoRV and XhoI, releasing the insert. The

insert is then isolated and ligated to pCDM7-Amp which had been similarly cut. The resulting product is used to transform *E. coli* DH5 α , from which larger quantities of plasmid nucleic acid may be isolated.

COS-7 (ATCC No. CRL 1651) cells are then transfected with pCDM7-Amp containing CRF₂ receptor cDNA (10 μ g nucleic acid/10 cm plate of cells) utilizing 400 μ g/ml of DEAE-Dextran and 100 μ M chloroquine. The cells are transfected for 4 hours, then shocked with 10% DMSO for 2 minutes. The cells are then washed, and grown in DMEM containing 10% Fetal Bovine Serum for 2 days in a 24-well plate.

EXAMPLE 3

CRF₂ RECEPTOR BINDING ASSAY

Materials

¹²⁵I-Tyr⁰-ovine CRF (¹²⁵I-oCRF; specific activity, 2200 Ci/mmol) is obtained from Du Pont-New England Nuclear (Boston, MA). Unlabeled rat/human CRF is purchased from Peninsula Laboratories (Belmont, CA). All other standard reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

A. Cell Membrane Preparation

Transfect cells which express CRF₂ receptors are harvested, washed once in PBS (pH 7.0 at 22°C), pelleted by centrifugation and stored at -70°C until use. On the day of assay, frozen pellets are resuspended in 5 ml of ice cold buffer containing 50 mM Tris, 10 mM MgCl₂, 2 mM EGTA pH 7.0 at 22°C and homogenized using a Polytron (PT3000, Brinkmann Instruments, Westbury, NY) at 27,000 rpm for 20 s. The homogenate is centrifuged at 48,000 x g for 10 min at 4°C, the supernatant discarded, and the pellet was re-homogenized in the same volume of buffer and centrifuged again at 48,000 x g for 10 min at 4°C. The resulting pellet containing membranes is resuspended in the above buffer to yield a final protein concentration of 300 mg/ml for use in the assay described below. Protein determinations were

performed according to the method of Lowry et al. (*J. Biol. Chem.* 193:265-275, 1951) using bovine serum albumin (BSA) as a standard.

B. CRF Receptor Binding Assay

One hundred microliters of the membrane suspension is added to 1.5 ml (polypropylene microfuge tubes) containing 100 μ l of (100-200 pM) 125 I-oCRF in incubation buffer (50 mM Tris, 10 mM MgCl_2 , 2 mM EGTA, 1.5% BSA, 0.15 mM bacitracin and 1.5% aprotinin) and 100 μ l of the incubation buffer. Competing peptides (e.g., r/hCRF, sauvagine, urotensin I, urotensin II, bovine CRF (b-CRF), and deamidated bovine CRF (b-CRF-OH)) are also added. Incubations are carried out at room temperature (22°C) for 2 hours, and terminated by centrifugation in a microfuge for 5 min at 12,000 x g. The resulting pellet is washed gently with 1.0 ml of ice-cold phosphate-buffered saline, pH 7.2 containing 0.01% Triton X-100 and re-centrifuged for 5 min at 12,000 x g. The microfuge tubes are cut just above the pellet, placed into 12 x 75 mm polystyrene tubes, and monitored for radioactivity in a Packard Cobra II gamma counter at approximately 80% efficiency. The non-specific binding of 125 I-oCRF to membrane homogenates, is defined in the presence of 1 mM unlabeled CRF.

C. Saturation Curve Analysis

For saturation studies, 100 μ l 125 I-oCRF (50 pM - 10 nM final concentration), 100 μ l of assay buffer (with or without 1 μ M r/hCRF final concentration, to define the non-specific binding) and 100 μ l of membrane suspension (as described above) are added in sequence to 1.5 ml polypropylene microfuge tubes. All reactions are carried out for 2 h at 22°C and terminated by centrifugation for 5 min at 12,000 x g. Aliquots of the supernatant are collected to determine the amount of unbound radioligand. The remaining supernatant is aspirated. Pellets are washed gently with ice-cold PBS plus 0.01% Triton X-100, centrifuged again, and monitored for bound radioactivity. Data from saturation curves are analyzed using the non-linear least-squares curve-fitting program LIGAND (Munson and Rodbard, *Anal. Biochem* 107:220-

229, 1980). Non-specific binding is not defined arbitrarily by the investigator, but rather is estimated as an independent variable from the entire data set.

D. Competition Curve Analysis

For competition studies, 100 μ l 125 I-oCRF (final concentration 200 - 300 pM) is incubated along with 100 μ l buffer (in the presence of 1 pM to 10 mM of competing ligands) and 100 μ l of membrane suspension as prepared above. The reaction is allowed to proceed for 2 h at 22°C and was terminated by centrifugation as described above. Data from competition curves were also analyzed by the program LIGAND. For each competition curve, estimates of the affinity of the radiolabeled ligand for the CRF receptor (125 I-oCRF or 125 I-r/hCRF) are obtained in independent saturation experiments. These estimates are constrained during the analysis of the apparent inhibitory constants (K_i) for the various related and unrelated peptides tested. Routinely, the data are analyzed using a one and two-site model comparing the "goodness of fit" between the models in order to accurately determine the K_i . Statistical analyses provided by LIGAND allowed the determination of whether a single-site or multiple-site model should be used. For CRF-related peptides, all data fit a single site model suggesting that the transfected cells contained a single homogeneous population of binding sites with high affinity and the appropriate pharmacological profile of the CRF receptor.

EXAMPLE 4

CAMP ASSAY

The effect of CRF and related peptides on adenylate cyclase activity in transfected cells may be determined essentially as follows. For agonist testing, compounds are added to wells with buffer only in order to identify compounds with intrinsic activity. For antagonist testing, compounds are placed in the wells along with 1 - 100 nM CRF to stimulate the adenylate cyclase system. Compounds are then assessed for their ability to inhibit the CRF-stimulated cAMP production in the

transfected cells. Briefly, cells are plated and grown in DMEM containing 10% fetal bovine serum for 2 - 4 days in 24-well plates at 37°C. On the day of assay, the medium is removed by vacuum aspiration and 100 µl of DMEM, 10 mM MgCl₂, 1 mM isobutylmethylxanthine (a phosphodiesterase inhibitor to inhibit the breakdown of cAMP produced) and 0.1% BSA (pH 7.2) are added to each well. CRF, related peptides and organic test molecules are added to individual wells. The plates are incubated at 37°C for a period of 1 hour. Following incubation, the wells are aspirated, rinsed once with PBS and aspirated again. 300 µl of 95% Ethanol and 20 mM HCl (EtOH/HCl) are then added to each well and the plates incubated at -20°C overnight to extract the produced cAMP. The EtOH/HCl is removed, placed in 1.5 ml polypropylene eppendorf tubes and the wells washed with an additional 200 µl of EtOH/HCl and pooled with the initial sample. All samples are dried in a Speed-Vac (Savant Instruments, Farmingdale NY) and either stored at -20°C until use or reconstituted with 500 µl of sodium acetate buffer pH 7.5 and assayed immediately for cAMP concentration using a radioimmunoassay kit from Biomedical Technologies Inc. (Stoughton MA) according to manufacturers instructions.

Figure 3 represents cAMP accumulation in cells transfected with the CRF₁ receptor and stimulated with ovine CRF (oCRF), rat/human CRF (r/hCRF), sauvagine or urotensin I. Briefly, this figure shows a dose-dependent increase in cAMP in response to these compounds. All of these compounds showed similar potencies. In Figure 4, the rat CRF_{2α} receptor has been transfected into cells and cAMP accumulation is measured in response to the same drugs as shown in Figure 3. As shown in Figure 4, sauvagine and urotensin I are more potent than either oCRF or r/hCRF at stimulating cAMP. Together, these findings show a clearly distinct pharmacological profile between the CRF₁ and CRF₂ receptors.

Figure 5 and Figure 6 depict the effect in CRF₁ and CRF_{2α} transfected cells, respectively, of the antagonists α-helical oRCF(9-41) and d-Phe r/hCRF(12-41) upon Sauvagine-stimulated cAMP production.

The above results have been formulated below in Table I, wherein the effective concentration of one-half maximal adenylate cyclase stimulation (EC_{50}) are presented.

Table I

PEPTIDE	human CRF_1 receptor	human $CRF_{2\alpha}$ receptor	rat $CRF_{2\beta}$ receptor
oCRF	10 nM	80 nM	N/A
r/hCRF	4 nM	20 nM	1 nM
Sauvagine	3 nM	0.5 nM	0.4 nM
Urotensin I	4 nM	2 nM	N/A
bCRF	30 nM	100 nM	N/A
bCRF-OH	1000 nM	>1000	N/A
Gly41 r/hCRF-OH	800 nM	>1000	N/A
r/hCRF(6-33)	>1000	>1000	N/A
d-Phe r/hCRF(12-41)	>1000	>1000	N/A
α -helical oCRF(9-41)	>1000	>1000	N/A
VIP	>1000	>1000	N/A
AVP	>1000	>1000	N/A
hGRF	>1000	>1000	N/A

EXAMPLE 5

TISSUE DISTRIBUTION OF CRF_2 α AND β RECEPTORS

In order to determine anatomical distribution of the two CRF_2 receptor forms, mRNA expression patterns were analyzed in isolated RNA (RNase protection assays) and whole tissue sections (*in situ* hybridization) from rat brain and peripheral tissues essentially as described below.

A. RNase Protection Assay

A 366-base riboprobe, containing 258-base antisense sequence specific to rat the CRF_{2α} receptor, and a 278-base riboprobe containing 181-base antisense sequence specific to the CRF_{2β} receptor mRNA were generated by T3 RNA polymerase in 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 U/μl of RNasin (Promega), 0.5 mM each of ATP, CTP, TTP, and 3 μM of ³²P-UTP (DuPont, 800 Ci/mmol), 0.1 μg/μl of template DNA, 1 U/μl of T3 RNA polymerase in total volume of 10 μl at 37°C for 30 minutes. A 712-base riboprobe containing 632-base of antisense sequence specific to rat β-actin mRNA was generated in the same conditions, except using T7 RNA polymerase. The probe synthesis mixtures were then treated with DNase I at concentration of 1 U/μl at 37°C for 10 minutes and then electrophoresed on a 5% denaturing acrylamide gel. The ³²P-labeled RNA probes with expected sizes were recovered from the gel with an RNA purification kit, RNaid kit (Bio-101, La Jolla, CA). RNA hybridization, RNase digestion and separation of protected RNAs were performed as described by Sawbrook et al., *supra*. In assays for CRF₂ receptor mRNAs, 40 μg of total RNA was used in each sample. In assays for β-actin mRNA, 1 μg of total RNA was used for each sample.

B. In Situ Hybridization

Male Sprague-Dawley rats were sacrificed by decapitation, and the brains removed and frozen in liquid isopentane (-42°C). Subsequently, tissues were sectioned (15 μm) on a cryostat maintained at -20°C and thaw mounted onto polylysine-coated microscope slides. Sections were stored at -80°C prior to tissue fixation.

Sections were removed from storage at -80°C and placed directly into 4% buffered paraformaldehyde at room temperature. After 60 minutes, slides were rinsed in isotonic phosphate buffered saline (10 minutes) and treated with proteinase K (1 μg/ml) in 100 mM Tris/HCL, pH 8.0) for 10 minutes at 37°C. Subsequently, sections underwent successive washes in water (1 minute), 0.1 M triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 minutes and 2X SSC (0.3 mM NaCl, 0.03 mM sodium

citrate, pH 7.2) for 5 minutes. Sections were then dehydrated through graded alcohols and air dried.

RNA probes (as described above) were synthesized using Maxiscript RNA transcription kits (Ambion, Austin, TX). Post-fixed sections were hybridized with 1.0×10^6 dpm of [^{35}S]UTP-labeled riboprobes in hybridization buffer containing 75% formamide, 10% dextran sulphate, 3X SSC, 50 mM sodium phosphate buffer (pH 7.4), 1X Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol in a total volume of 25 μl . The diluted probe was applied to sections on a glass coverslip which was sealed into place with rubber cement. Sections were hybridized overnight at 55°C in a humid environment.

Post-hybridization the rubber cement was removed and sections were washed in 2X SSC for 5 minutes and then treated with RNase A (200 $\mu\text{g/ml}$ in 10 mM Tris/HCL, pH 8.0, containing 0.5 M NaCl) for 60 minutes at 37°C. Subsequently, sections were washed in 2X SSC for 5 minutes, 1X SSC for 5 minutes, 0.5X SSC for 60 minutes at hybridization temperature, 0.5X SSC for 60 minutes at room temperature for 5 minutes and then dehydrated in graded alcohols and air dried. For signal detection, sections were placed on Kodak XAR-5 X-ray film and exposed for 7 days at room temperature.

C. Results

As shown in Figure 7, based upon RNase protection assays CRF $_{2\alpha}$ and CRF $_{2\beta}$ receptor mRNAs have clearly distinct tissue distributions. In particular, CRF $_{2\alpha}$ is found primarily in the brain, particularly in the hypothalamus, lateral septum and olfactory bulb, whereas the CRF $_{2\beta}$ mRNA is found primarily, but not restricted to, the heart and skeletal muscle. This is in stark contrast to the previously disclosed distribution of the CRF $_1$ receptor (Potter et al., *PNAS* 91:8777-8781, 1994).

Results of *in situ* hybridization assays are shown in Figure 8. Figures 8A, B, C and 8A', B', C' show coronal sections of rat brain probed with antisense CRF $_{2\alpha}$ and CRF $_{2\beta}$ probes, respectively. There is clear expression of CRF $_{2\alpha}$ in the lateral septum

(A), whereas CRF_{2β} is expressed in choroid plexus (A'). Figures 8B and 8C show CRF_{2α} expression in the paraventricular nuclei and ventromedial hypothalamic nuclei, respectively. CRF_{2β} is not detected in either of these areas (Figures 8B' and 8C'). CRF_{2α} is also detected in the supraoptic nuclei (Figure 8B), whereas CRF_{2β} is expressed in the adjacent arterioles (Figure 8B'). CRF_{2α} was also found to be expressed on many of the arterioles in the brain which express CRF_{2β} albeit at a lower level (not shown). In heart, CRF_{2β} was the major form expressed, and was found predominantly on arterioles (not shown).

EXAMPLE 6

ANATOMICAL DISTRIBUTION OF CRF₁ AND CRF₂ mRNA

A. Materials and Methods

1. *Animals*: Male Sprague Dawley rats (200-220 g) were used for mapping studies. Prior to sacrifice, animals were housed in a 12 hr light/dark cycle with food and water provided *ad libitum*.

2. *Riboprobe Design*: CRF₂ cRNA riboprobe was produced from a 460 bp cDNA fragment of the CRF₂ receptor sub-cloned into pBluescriptSK+ (Stratagene, La Jolla) and linearized with *Xba* I. CRF₁ probe was synthesized from a 460 bp 5' fragment of CRF₁ cDNA in pBluescriptSK+, linearized with *Xba* I. Both CRF₂ and CRF₁ riboprobes were directed against the 5' region of their respective receptors, covering the sequence up to the third presumed transmembrane region (Figure 9). The approximate nucleotide homology between the two probes is 65% in this region. Importantly, in preliminary experiments, cRNA probes directed against the 3' region of the CRF₂ receptor apparently labeled both CRF₁ and CRF₂ receptor mRNA's whereas the two mRNA species could be clearly separated by 5' specific probes under similar hybridization conditions. Thus, it seems necessary to utilize 5' probes for subtype-specific labeling. For both probes, specificity was confirmed by absence of signal in sections labeled with sense probe and sections pretreated with RNase prior to

hybridization with antisense (cRNA) probe. CRF cRNA antisense probes were synthesized from a 770 bp fragment of CRF cDNA sub-cloned into a pGEM3Z vector (courtesy Dr. Robert Thompson, University of Michigan). Riboprobes were produced using either T3 or T7 transcription systems in a standard labelling reaction mixture consisting of: 1 µg linearized plasmid, 5X transcription buffer, 125 µCi ³⁵S-UTP or ³³P-UTP, 150 µM NTP's, 12.5 mM dithiothreitol, 20U RNase inhibitor and 6U of the appropriate polymerase. The reaction was incubated at 37°C for 90 min, labeled probe being separated from free nucleotides over Sephadex G-50 spin columns.

3. *In Situ Hybridization Histochemistry*: Dissected tissue was frozen in isopentane cooled to -42°C and subsequently stored at -80°C prior to sectioning on a cryostat. Slide-mounted tissue sections were then stored at -80°C. Sections were removed from storage and placed directly into 4% buffered paraformaldehyde at room temperature. After 60 min, slides are rinsed in isotonic phosphate buffered saline (10 min) and treated with proteinase K (1 µg/ml in 100 mM Tris/HCl, pH 8.0) for 10 min at 37°C. Subsequently, sections underwent successive washes in water (1 min), 0.1 M triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 min and 2X SSC (0.3 mM NaCl, 0.03 mM sodium citrate, pH 7.2) for 5 min. Sections were then dehydrated through graded alcohols and air dried. Post-fixed sections were hybridized with 1.0×10^6 dpm [³⁵S]UTP-labelled riboprobes in hybridization buffer containing 75% formamide, 10% dextran sulphate, 3X SSC, 50 mM sodium phosphate buffer (pH 7.4), 1X Denhardt's solution, 0.1 mg/ml yeast tRNA and 10 mM dithiothreitol in a total volume of 30 µl. The diluted probe was applied to sections on a glass coverslip and hybridized overnight at 55°C in a humid environment. Post-hybridization, sections were washed in 2X SSC for 5 min and then treated with RNase A (200 µg/ml in 10 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl) for 60 min at 37°C. Subsequently, sections were washed in 2X SSC for 5 min, 1X SSC for 5 min, 0.1X SSC for 60 min at 70°C, 0.5 X SSC at room temperature for 5 min and then dehydrated in graded alcohols and air dried. For signal detection, sections were placed on Kodak Bio Max X-ray film and exposed for the required length of time or dipped in photographic emulsion (Amersham LM-1) for high

resolution analysis. Autoradiograms were analyzed using automated image analysis (DAGE camera/Mac II/ IMAGE program). Briefly, anatomical regions of interest were interactively selected and mean optical density measurements determined from at least three coronal sections. Background signal was determined from an area of section in which labelling was undetectable. Dipped sections were examined using a Zeiss Axioscope.

B. Results

1. CRF₂ Probe Selection

As illustrated in Figure 1, the CRF₂ cRNA probe utilized in the present studies was synthesized from a 460 bp 5' fragment of CRF₂ cDNA. Preliminary *in situ* hybridization studies using cRNA probes encompassing the 3' portion of the receptor (bearing high homology to the CRF₁ receptor) afforded an anatomical distribution which was inconsistent with that obtained using 5'-specific riboprobes. The pattern of labelling was, however, consistent with *both* CRF₁ and CRF₂ receptor mRNA distribution. The high sequence homology between CRF₁ and CRF₂ receptors thus necessitates the use of 5'-containing riboprobes for specific hybridization of CRF₂ or CRF₁ receptor subtype mRNA.

2. Anatomical Distribution

The comparative anatomical distribution of CRF₂ and CRF₁ mRNA was determined in adjacent coronal brain sections. Table II below summarizes the semi-quantitative analysis of the data.

As illustrated in horizontal section (Figure 10), the distribution of CRF₂ receptor mRNA clearly differs from that of CRF₁, exhibiting a distinct sub-cortical pattern. While CRF₁ mRNA expression was high in a range of telencephalic structures, CRF₂ receptor expression exhibited a more anatomically specific pattern including the lateral septal region (LS), the bed nucleus of the stria terminalis (BNST), the

amygdaloid area and the olfactory bulb (Figure 11, Table 1). The contrast in expression patterns between CRF receptor subtypes was particularly evident within the septal region: CRF₂ mRNA expression was very high in the lateral septal nuclei but very low in the medial septum, the septal nucleus where CRF₁ mRNA abundance was most evident (Figure 11B). The distribution of CRF₂ receptor mRNA within the LS was, however, heterogeneous; very high levels of expression were evident within both the intermediate and ventral sub-nuclei with only an occasionally labelled cell evident in the dorsal sub-division (Figure 12). Within both the intermediate and ventral regions of the LS the level of CRF₂ receptor expression exhibited an apparent rostro-caudal gradient, with a smaller percentage of labelled cells detected in the caudal aspects of both areas. At this level, some scattered CRF₂-expressing cells were also evident in both the vertical and horizontal limbs of the diagonal band. Again, contrasting the higher levels of CRF₁ mRNA found in these regions (Figure 11). Differential patterns of CRF receptor subtype expression were also evident within the olfactory bulb (Figure 11A). Here, CRF₁ receptor expression was particularly high in the internal granule cell and mitral cell layers with lower levels detectable in the external granule layer and ependyma. However, most cells expressing CRF₂ receptors were found lining the ventricle within the ependymal area and the internal granule cell layer (Figure 11A). Both CRF₁ and CRF₂ receptors were also expressed in the accessory olfactory nucleus.

Both CRF₂ and CRF₁ receptor mRNA expression were evident in the BNST, particularly in the medial aspect, and the amygdaloid area. Within the BNST, CRF₂ receptor expression appeared to be lower in the lateral regions where the highest abundance of CRF-expressing cells were found (Figures 13A and B). CRF₁ receptor expression was found in both medial and lateral divisions of the BNST (Figure 13C). Within the amygdala the highest levels of CRF₂ receptor expression were evident in the cortical and medial amygdaloid nuclei with less expression in the basolateral nucleus (Figure 11C). In complementary fashion, CRF₁ receptor expression was very high in the basolateral area but low in the cortical amygdala (Figure 14). Both CRF₁ and CRF₂ receptor mRNA expression were unremarkable in the central nucleus. Within the

hippocampal formation, CRF₂ receptors were generally expressed in low to moderate levels in pyramidal cells of CA subfields and granule cells of the dentate gyrus. However, scattered cells with high levels of CRF₂ expression were evident in non-granule cell layers in ventral dentate gyrus (Figure 15). CRF₁ receptor expression was most abundant in the pyramidal cell layer of CA_{3/4} with moderate levels evident in CA₁. However, CRF₁ receptor expression was apparently absent in the dorsal dentate gyrus (Figure 15). Interestingly, higher levels of CRF₁ expression were evident in the ventral hippocampus compared to the dorsal aspect (Figure 11C and D). Cells expressing both CRF₁ and CRF₂ were found throughout entorhinal cortex.

The distribution of CRF₂ receptor transcripts in diencephalic structures was confined mainly to the hypothalamus where labelled cells were evident in preoptic, anterior and tuberal regions. The levels of CRF₂ mRNA expression found in the ventromedial hypothalamic nuclei (VMH) were amongst the highest detected in brain (Figure 16). Both dorsal and ventral aspects of VMH displayed high levels of CRF₂ mRNA relative to other brain regions although CRF₂ mRNA expression was most evident in the dorsomedial division. At this level, however, CRF₁ receptor expression was predominantly localized to the dorsomedial hypothalamic nucleus (DMH) with only a limited number of cells labelled in the VMH. In anterior hypothalamus, CRF₂ mRNA was localized to highly expressing cells in the supraoptic nucleus (SO) and medial areas of the paraventricular nucleus (PVN) (Figure 17, Figure 18). Within the PVN, CRF₂ mRNA appeared to be expressed in medial parvocellular cells, partially coinciding with CRF mRNA expression (Figure 10A). This raises the interesting possibility that CRF₂ receptors may act as autoreceptors in selective cells in this nucleus. In both the SO and PVN, the number of cells expressing CRF₁ mRNA was extremely low (Figure 17, Figure 18). Cells expressing CRF₂ mRNA were also evident throughout the anterior and lateral hypothalamic areas, the suprachiasmatic nucleus and in the medial preoptic area (MPA).

In the midbrain, CRF₂-expressing cells were evident throughout nuclei biochemically characterized as serotonin-containing nuclei. Strongly hybridizing cells

were localized along the midline in the dorsal raphe nucleus (DR), the caudal linear nucleus, and in the lateral aspects of the central gray (Figure 19). More ventrally, CRF₂ receptor expression was also detectable in the median raphe (MR) and the interpeduncular nucleus (IPN), especially within the rostral subnucleus (Figure 19). With the exception of IPN, CRF₁ receptor mRNA levels were generally low in all of these areas. At this level, CRF₂-expressing cells were also evident in clusters in the lateral and dorsal regions of the interior colliculus. However, higher levels of CRF₁ mRNA expression was also present in the inferior colliculus. However, higher levels of CRF₁ mRNA were found in the visually-receptive fields of the superior colliculus where CRF₂ receptor expression was very low. This differential expression pattern was repeated in other sensory relay structures of the brainstem. Thus, while CRF₁ receptor mRNA abundance levels were very high in tegmental nuclei and sensory trigeminal areas, CRF₂ receptor expression was limited to a few scattered cells in these structures (Table II). Similarly, within the cerebellum, CRF₁ receptor mRNA levels were very high in Purkinje and granule cell layers while CRF₂ receptor mRNA was present at very limited levels in granule cells (Figure 11E).

Within non-neuronal structures, a very high level of CRF₂ receptor expression was evident in the choroid plexus of the third, fourth and lateral ventricles (Figure 20). In addition, cerebral arterioles consistently exhibited CRF₂ mRNA at all brain levels examined (Figure 21). CRF₁ mRNA was detectable at near background levels in these structures. In the pituitary gland, CRF₁ expression was detectable in both anterior and intermediate lobes with particularly high expression in clusters within the anterior lobe (Figure 22). Within the anterior lobe, CRF₂ receptor expression was detectable only in scattered cells (Figure 22).

C. Discussion

The present *in situ* hybridization studies indicate that at least two CRF receptor subtypes, CRF₁ and CRF₂, are expressed in mammalian rat brain. The heterogeneous anatomical distribution patterns of CRF₁ and CRF₂ mRNA expression

suggests distinct functional roles for each receptor in CRF-related CNS circuits. While CRF₁ receptor expression was most abundant in neocortical, cerebellar and sensory relay structures, CRF₂ receptor expression was generally localized to specific sub-cortical structures, including the lateral septum and various hypothalamic nuclei.

Within the forebrain, the highest levels of CRF₂ receptor expression were found in the lateral septal nuclei. The lateral septum, by virtue of widespread reciprocal connections throughout the brain, is implicated in a variety of physiological processes, which range from higher cognitive functions such as learning and memory to autonomic regulation, including food and water intake. In addition, the septum plays a central role in classical limbic circuitry and is thus important in a variety of emotional conditions including fear and aggression. The lateral septal nuclei receive CRF-containing afferents from rostral hypothalamic regions, particularly the anterior hypothalamic area (Sakanaka et al., *J. Comp. Neurol.* 270:404-415, 1988). Interestingly, the majority of these CRF-like immunoreactive fibres are found in the most lateral aspects of the LSV and in the LSI (Sakanaka et al., *J. Comp. Neurol.* 270:404-415, 1988), septal subnuclei exhibiting the highest levels of CRF₂ receptor mRNA (Figure 12). The lack of CRF₁ receptor expression in these nuclei suggests that CRF₂ receptors may solely mediate the postsynaptic actions of CRF inputs to this region. The principal GABAergic neurons of the lateral septum provide inhibitory input to hypothalamic regions, as well as amygdaloid nuclei (Jakab et al., 1991, *Calbindin-containing somatospiny neurons of the rat lateral septal area project to the medial amygdala and the anterior hypothalamus*, Third IBRO World Congress Neuroscience Abstracts, 324) and receive excitatory glutamatergic input from the hippocampal formation (Joels and Urban, *Experimental Brain Research* 54:455-462, 1984). The lateral septum thus acts as both an integrator of limbic circuitry and an interface between telencephalic and diencephalic areas. The high level of CRF₂ receptor expression in this area indicates that CRF₂ receptors can modulate limbic circuitry at the level of septal activity.

In agreement with previous *in situ* hybridization studies (Potter et al., *Proceedings of the National Academy of Sciences* 91:8777-8781, 1994), a general lack

of CRF₁ receptor expression in hypothalamic nuclei was noted. From the present studies it is clear that CRF₂ receptor mRNA was evident throughout the rostro-caudal extent of the hypothalamus while CRF₁ receptor expression was limited. The difference in CRF receptor subtype expression levels was particularly evident within the paraventricular nucleus where CRF₂ receptor expression was readily detectable while CRF₁ receptor mRNA was present only in scattered cells. The distribution of cells expressing CRF₂ receptor mRNA within the PVN coincides with the cellular distribution of CRF mRNA (Figure 18) indicating an autoreceptor role for CRF₂ receptors in this nucleus. The CRF neurons of the PVN play a classical hypophysiotropic role in controlling ACTH release from the anterior pituitary (Wiegand and Price, *J. Comp. Neurol.* 192:1-19, 1980) and as such are central to the control of the mammalian hypothalamo-pituitary-adrenal system. In addition to this stress axis-related role, subpopulations of PVN CRF neurons, particularly within the dorsal parvocellular region and ventral aspect of the medial parvocellular region (mpv), project to autonomic cell groups in the brainstem and spinal cord (Sawchenko, *Brain Res.* 437, 1987). Thus, the high level of CRF₂ receptor expression within the mpv suggests a presynaptic role for CRF₂ receptors in modulating autonomic-related CRF projection neurons.

A selective expression of CRF₂ receptor mRNA was also evident in the magnocellular neurosecretory neurons of the supraoptic nucleus. In view of the putative association of CRF₂ receptor expression with CRF neurons in the PVN, it is relevant that a subset of SO neurons also synthesize CRF (Kawata et al., *Cell Tissue Research* 230:239-246, 1983). The presence of CRF₂ receptors in both SO and PVN neurons indicates that these sites may act to influence hypothalamic input to both the anterior and posterior pituitary. Within the pituitary, however, CRF₁ receptor expression predominates over CRF₂ expression in both the intermediate and anterior lobes. Thus, in terms of HPA axis activity, CRF₂ receptors may mediate CRF effects at the level of the hypothalamus while CRF₁ receptors are responsible for CRF-induced changes in ACTH release in pituitary corticotropes.

Within the caudal hypothalamus, CRF₁ and CRF₂ receptors exhibited mutually exclusive patterns of mRNA distribution: CRF₁ receptor mRNA being abundant in the dorsomedial nucleus but low within the VMH, while CRF₂ receptor mRNA expression was very high within the VMH but undetectable within the DMH. CRF-containing fibres originating in the corticomedial amygdala and subiculum terminate within the VMH (Sakanaka et al., *Brain Res.* 382:213-238, 1986). Afferents from the VMH in turn innervate the septum, BNST and amygdala, as well as brainstem regions (Simerly, R.B., Anatomical substrates of hypothalamic integration. In: *The Rat Nervous System* (Paxinos, G., eds.), pp. 353-376, Academic Press, 1995). Microinjection of CRF into the VMH is associated with changes in both autonomic outflow and gastrointestinal function (Brown and Fisher, Regulation of the autonomic nervous system by corticotropin-releasing factor. In: *Corticotropin-releasing factor: Basic and clinical studies of a neuropeptide* (De Souza, E.B., Nemeroff, C.B., eds.), pp. 291-298, Boca Raton, FL, CRC Press, Inc., 1990; Tache et al., CRF: Central nervous system action to influence gastrointestinal function and role in the gastrointestinal response to stress. In: *Corticotropin-releasing factor: Basic and clinical studies of a neuropeptide* (De Souza, E.B., Nemeroff, C.B., eds.), pp. 299-308, Boca Raton, FL, CRC Press, Inc., 1990). The high level of CRF₂ receptor expression in the VMH implicates this CRF receptor subtype as a terminal or somato-dendritic regulator of these CRF-related physiological functions. Moreover, dysregulation of CRF₂ receptors in this locus, or the PVN, may participate in the proposed role of central CRF systems in the development of obesity/anorectic syndromes (Krahn and Gosnell, *Psychiatric Medicine* 7:235-245, 1989).

In addition to CRF involvement in the development of eating disorders, a large body of evidence exists to implicate this neuropeptide in the pathophysiology of affective diseases such as anxiety and depression. For example, CRF injected into the rodent locus coeruleus produces an anxiogenic response while benzodiazepine anxiolytics have been shown to reduce CRF concentrations in the same nucleus (Owens et al., *J. Pharmacol. Exp. Ther.* 258:349-356, 1991). In clinical studies of major

depression, patients have been found to exhibit signs of CRF hypersecretion including: increased CRF concentrations in CSF, increased HPA activity, a blunted ACTH response to CRF and pituitary and adrenal hypertrophy (Owens and Nemeroff, The role of corticotropin-releasing factor in the pathophysiology of affective and anxiety disorders: laboratory and clinical studies. In: Corticotropin-Releasing Factor (Chadwick, D.J., Marsh, J., Ackrill, K., eds.), pp. 296-316, John Wiley and Sons, 1993). In view of the stimulatory role of CRF in HPA activity, it remains possible that the hypercortisolemia observed in depression results from increased central CRF drive. The possibility that hyperactivity of brain CRF circuits may contribute to the symptomatology of depressive illness is supported by preclinical studies in both rodents and nonhuman primates. In both species, central administration of CRF produces a spectrum of behavioral responses reminiscent of human depressive illness (Koob and Britton, Behavioral effects of corticotropin-releasing factor. In: Corticotropin-releasing factor: Basic and clinical studies neuropeptide (De Souza, E.B., Nemeroff, C.B., eds.), Boca Raton, FL, CRC Press, Inc., 1990; Kalin, Behavioral and endocrine studies of corticotropin-releasing hormone in primates. In: Corticotropin-releasing factor: Basic and clinical studies of a neuropeptide (De Souza, E.B., Nemeroff, C.B., eds.), pp. 275-290, Boca Raton, FL, CRC Press, Inc., 1990). While the specific underlying mechanisms by which CRF evokes such behavioral responses remain largely undefined, it is likely that modulation of brain limbic circuitry and the participation of specific populations of CRF neurons are involved (Rainnie et al., *J. Pharm. Exp. Therap.* 263:846-858, 1992). In this regard, the present study provides an anatomical basis for the involvement of *both* CRF₁ and CRF₂ receptors in mediating limbic CRF effects. While the CRF₂ receptor may be regarded as the predominant hypothalamic CRF receptor, both CRF₁ and CRF₂ receptors were localized to classical limbic structures such as the amygdaloid complex, the hippocampus and the septal nuclei.

In addition to neuroendocrine abnormalities, a convincing body of data indicates dysfunction in central serotonergic activity in depressive illness. From postmortem studies, it is clear that serotonin metabolism and specific receptor subtypes

are altered in some brain regions in depressed patients (Meltzer, *Br. J. Psychiat.* 155:25-31, 1989; Yates et al., *Psychiatry* 27:489-496, 1990). Drugs which inhibit serotonin metabolism, inhibit serotonin uptake from the synapse or act to directly stimulate serotonin receptors are all effective antidepressants (Peroutka and Snyder, *Science* 210:88-90, 1980; Traber and Glaser, *Trends Pharmacol. Sci* 8:432-437, 1987). Thus as both the HPA axis and serotonergic system are implicated in affective disease, it is likely that interactions between these two systems may be relevant to the pathophysiology and pharmacotherapy of depression (Chalmers et al., *Clin. Neurosci.* 1:122-128, 1993). In this context, the present data indicates a selective expression of CRF₂ receptor mRNA in midbrain serotonergic cell body nuclei. Both dorsal and median raphe nuclei exhibited CRF₂ mRNA, as well as cells in serotonin-associated regions of the interpeduncular nucleus and central grey (Figure 19). As the raphe nuclei receive CRF-ergic input from forebrain regions (Sawchenko and Swanson, *Organization of CRF immunoreactive cells and fibers in the rat brain: immunohistochemical studies*. In: *Corticotropin-releasing factor: Basic and clinical studies of a neuropeptide* (De Souza, E.B., Nemeroff, C.B., eds.), pp. 29-52, Boca Raton, FL, CRC Press, Inc., 1990) any CRF-induced modulation of serotonergic activity is likely to be CRF₂ receptor mediated. Such interaction provides an anatomical and biochemical basis for central "stress"-related modulation of serotonergic function and a basis for theories of stress-induced affective disorders.

In addition to neuronal localization the present study also indicates a high level of CRF₂ receptor expression in both the choroid plexus and cerebral arterioles. The presence of signal in both structures may be indicative of an endothelial cell localization. The CRF₂ cRNA probe used in the present studies did not differentiate between the two apparent splice forms of the CRF₂ receptor, CRF_{2α}, and CRF_{2β} (Lovenberg et al., *Proc. Natl. Acad Sci USA* 92:836-840, 1995). However, preliminary data indicates that the CRF_{2β} form of the receptor may predominate in blood vessels. Interestingly, the CRF_{2β} receptor is also expressed in peripheral tissues such as heart, lung and skeletal muscle where it may act to mediate vascular effects of CRF.

In summary, a differential cellular distribution of CRF₂ and CRF₁ receptor mRNA has been identified in rat brain and pituitary gland. This distribution suggests that the CRF₁ receptor is the primary neuroendocrine pituitary CRF receptor and plays a dominant role in the cortical, cerebellar and sensory roles of CRF in brain. The regional anatomical distribution of CRF₂ receptor mRNA indicates a role for this receptor in relation to hypothalamic neuroendocrine, autonomic and behavioral actions of brain CRF. The presence of CRF₂ receptor mRNA in the hypothalamic PVN and medial and cortical amygdaloid regions may indicate an autoreceptor role for this site in selective circuits.

Table II

SEMIQUANTITATIVE EVALUATION OF CRF₁ AND CRF₂ RECEPTOR MRNA
DISTRIBUTION IN RAT BRAIN AND PITUITARY GLAND

Anatomical Region	CRF1 mRNA Abundance	CRF2 mRNA Abundance
<i>Telencephalon:</i>		
Olfactory Bulb:		
External Granular Layer	++	-
Internal Granular Layer	++++	++
Mitral Cell Layer	++++	-
Ependyma	++	+++
Accessory Olfactory	++	++
Nucleus		
Frontal Cortex (Superficial)	+++	-
Frontal Cortex (Deep)	+++	-
Cingulate Cortex (Superficial)	+++	-
Cingulate Cortex (Deep)	+++	-
Lateral Septum (Ventral)	+	++++
Lateral Septum (Intermediate)	+	++++

Anatomical Region	CRF1 mRNA Abundance	CRF2 mRNA Abundance
Medial Septum	++	-/+
Bed Nucleus of the Stria	++	++
Terminalis (Medial)		
Amygdala:		
Basolateral Nucleus	++++	-/+
Medial Nucleus	++++	++
Posterior Cortical	+	+++
Hippocampus:		
CA1	++	++
CA3/4	++++	++
Dentate Gyrus	++	++
Entorhinal Cortex	++	++
<i>Diencephalon:</i>		
Hypothalamus:		
Paraventricular Nucleus	-/+	++
Supraoptic Nucleus	+	+++
Lateral Hypothalamus	+	+
Dorsomedial Hypothalamus	+++	-
Ventromedial Hypothalamic Nucleus	+	++++
Medial Geniculate Nucleus	++	-/+
<i>Mesencephalon:</i>		
Superior Colliculus (Superficial Layer)	+++	+
Interpeduncular Nucleus	++++	+++
Dorsal Raphe Nucleus	+	++
Caudal Linear Nucleus	+	+++
Red Nucleus	++++	-

Anatomical Region	CRF1 mRNA Abundance	CRF2 mRNA Abundance
<i>Pons/Medulla:</i>		
Inferior Colliculus	++	++
Lateral Dorsal Tegmental Nucleus	++++	-
Locus Coeruleus	-	-
Cerebellar Cortex	++++	-/+
Pontine Gray	++++	-/+
Motor Trigeminal Nucleus	++++	-
Sensory Trigeminal Nucleus	+++	-/+
Choroid Plexus	-	++++
<i>Pituitary Gland</i>		
Anterior Lobe	+++	-/+
Intermediate Lobe	+++	-/+
Posterior Lobe	-	-

CRF₁ and CRF₂ mRNA abundance for each anatomical region was determined from optical density measurements. Density values for each parameter are presented according to their respective percentile distributions: +++++ (>75%), very dense; +++ (<75%, >50%), dense; ++ (<50%, >25%), moderate; + (<25%, >10%), low; -/+ (<10%), scattered cells.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.